The effects of hypothermia and acidosis on Haemostasis

By

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration.

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Finally I would like to dedicate this Thesis to our gorgeous and beloved son, Oliver (Super Nené) and also to our future little girl Carlota.

Hypothermia and acidosis are often seen in trauma patients and the critically ill. Both significantly contribute towards the pathology of these conditions. These patients are often investigated for coagulopathy, often as a result of bleeding. Traditionally routine coagulation screening is performed in buffered systems at 37°C. Therefore the information returned by the laboratory tells the clinician what the clotting will be like if the hypothermia and acidosis are corrected. This is useful clinical information but does not accurately reflect the clinical situation at the time.

Blood was taken from 40 consenting healthy adults and 17 critically ill patients to examine the effects of hypothermia and acidosis upon “normal” clotting. The STart®4 coagulometer was used to assess the prothrombin time (PT) and the activated partial thromboplastin time (APTT), the ROTEM® analyzer to assess thromboelastometry and the Fluoroscan Ascent to assess thrombin generation using calibrated automated thrombography (CAT).

The results show a difference in interpretation between assays using fibrin polymerisation as an end-point as opposed to the generation of thrombin. The results of the CAT assay under hypothermic and/or acidotic conditions suggest that in spite of having initially a delayed lag time (lag time at 37°C = 7.91 min; lag time at 31°C = 8.37 min (Table 4.3.)), once the thrombin burst occurs it will generate more thrombin which will increase the overall ETP (ETP at 37°C = 1777nmol of thrombin; ETP at 31°C = 2681nmol of thrombin (Table 4.3.)). Therefore a thrombotic profile may be seen instead of a bleeding tendency.

In general, it seems that both parameters (lag time and ETP) are affected by both hypothermic and acidotic conditions on their own but when combining both of them together (hypothermia + acidosis) there was a cumulative effect which exaggerates the changes even more (lag time at 37°C/ pH 7.35 =.7.91min; lag time at 31°C/ pH 6.9 =.10.46min. and ETP at 37°C/ pH 7.35 =.1777nmol of thrombin; ETP at 31°C/ pH 6.9 =.2852 nmol of thrombin (Table 4.3.)).

These particular observations have not been observed previously in any published literature to date, where a hypocoagulable coagulopathy is described under abnormal conditions, such as hypothermia and acidosis.

Although these standard clotting tests are performed at 37°C and physiological pH, *in vitro* testing of any given sample should ideally be performed at the patient's own body temperature and pH as well as under the corrected conditions. This will give a picture of what is happening now compared to what will happen when the hypothermia/acidosis is corrected.

It is possible that global assays of haemostasis may offer some insight into the pathology of hypothermia and acidosis. However, as always in science many more questions are raised than are answered.

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5-hydroxytryptamine or 5-HT: Serotonin

A-aDO_2: alveolar-arterial oxygen tension difference

ADP: Adenosine diphosphate

APACHE: Acute Physiology, Age, Chronic Health Evaluation

APC: Activated Protein C

APTT: Activated Partial Thromboplastin Time

ATP: Adenosine triphosphate

AUC: Area under the Curve

BSA: Bovine Serum Albumin

cAMP: Cyclic adenosine monophosphate

Ca^{2+} : Calcium

CAT: Calibrated Automated Thrombography

CFT: Clot Formation Time

CT: Clotting Time

CTI: Corn Trypsin Inhibitor

DIC: Disseminated Intravascular Coagulation

DMSO: Dimethyl Sulphoxide

ETP: Endogenous Thrombin Potential

FIO_2 :Fraction of inspired oxygen

FDP: Fibrin Degradation Products

FV: Factor V

FVIII: Factor VIII

FIX: Factor IX

FX: Factor X

FXI: Factor XI

FXII: Factor XII

FXIII: Factor XIII

ICU: Intensive Care Unit

kDa: Kilo Dalton

LAG: Lag Time

MAXV: Maximum velocity

MAXV-t: Time to the Maximum Velocity

MCF: Maximum Clot Firmness

MK: Megakaryocyte

ML: Maximum lysis

PAI-1: Plasminogen activator inhibitor-1

PaO_2: partial oxygen arterial pressure

PAP: Preactivation Peptide

PARs: Protease-activated receptor

PC: Phosphatidyl-Choline

PE: Phosphatidyl-Ethanolamine

PEAK: Peak Height of the CAT Curve

PGI_2:Prostaglandin I_2

PPP: Platelet Poor Plasma

PRP: Platelet Rich Plasma

PS: Phosphatidyl-Serine

PT: Prothrombin Time

TEG: Thromboelastometry

TF: Tissue Factor

TPA: Tissue Plasminogen Activator

TPO: Thrombopoietin

TT: Thrombin Time

TXA2: Thromboxane A2

1. INTRODUCTION

1.1. Haemostasis

Haemostasis is the complex mechanism of blood clotting and the ability to dissolve the clot after the damaged tissue has been repaired. This system allows the body can to control blood loss when vascular injury occurs. Haemostasis involves several reactions which require enzymes and tissues, such as coagulation factors, platelets, endothelial cells, fibrinolytic proteins, collagen and subendothelial microfibrils.

First event when a vessel is damaged is vascular constriction. This will restrict the amount of blood that gets to the area of injury.

With some internal endothelium lost, the anticoagulant action of the endothelial cells is interrupted. Now collagen and subendothelial microfibrils are in contact with the blood components. Platelets, which circulate close to the vessel wall will locate and adhere to the injury site.

After platelet adhesion has commenced, platelet activation will follow. Activated platelets change shape and release their granule contents (thromboxane A_2, calcium, epinephrine, ADP and several coagulation factors). Activated platelets attract and activate other platelets and with the help of fibrinogen platelet aggregation starts to form a platelet plug.

At this stage, the polymerisation of fibrinogen to fibrin will form a mesh that transforms an unstable platelet plug in to solid clot.

When the vessel is finally repaired the clot is dissolved trough the fibrinolytic pathway, where plasmin plays the main role. Once the clot is removed blood flow is restored and vessel activity is back to normal.

1.1.1. Coagulation cascade

At the site of injury the subendothelial tissues will be exposed and initiate the coagulation cascade (Fig. 1.1). Tissue Factor (TF) activates factor VII (VIIa), which will activate Factor IX (FIXa) and and also factor X into factor Xa (Krupiczojc *et al*., 2008).

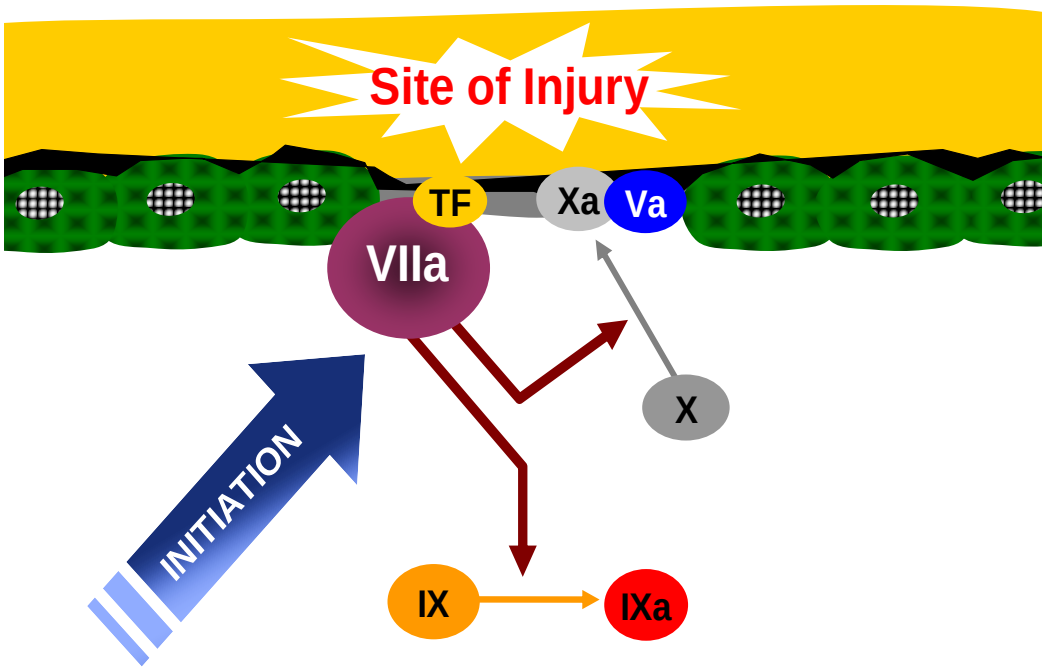


Figure 1.1. Coagulation Cascade Initiation

FXa helps to catalyze the conversion of prothrombin into thrombin.(Bode *et al*., 2006) thrombin is the main protein in the coagulation cascade; once thrombin is in the plasma the coagulation cascade changes dramatically. Thrombin has several functions (Davie *et al*., 2006).

For example, it will work as an agonist to further activate platelets via a membrane platelet receptor: the Protease-activated receptor (PAR). Further stimulation of coagulation occurs as a result of back activation of factors by Thrombin. The back activation of factor XI (FXI) results in more generation of FIXa.

The simultaneous activation of the cofactor FVIII allows the formation of the Tenase complex which generates significant quantities of FXa (Fig. 1.2).

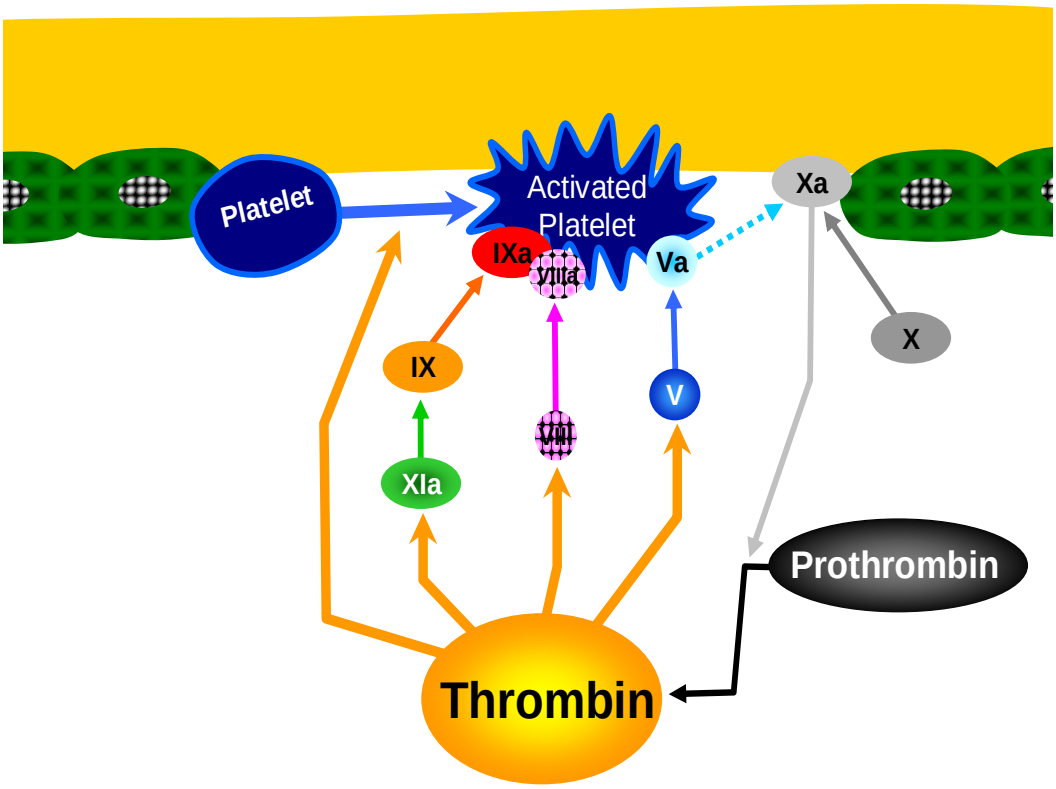


Figure 1.2. Thrombin is the most important constituent of the Coagulation Cascade.

Activation of another cofactor FV results in formation of the Prothrombinase complex (Swords *et al*., 1993) which results in an increase in thrombin generation, the Thrombin Burst. This is known as cascade amplification by thrombin.

The coagulation cascade is a complex process to ultimately form fibrin strands (Fig.1.3).
These fibrin strands form a mesh along with aggregated platelets and trapped red cells at the site of vascular injury to form a clot.

Finally, the clot firmness and stability is achieved by the action of activated factor XIII (FXIIIa) (Also activated by thrombin) on the fibrin strands.

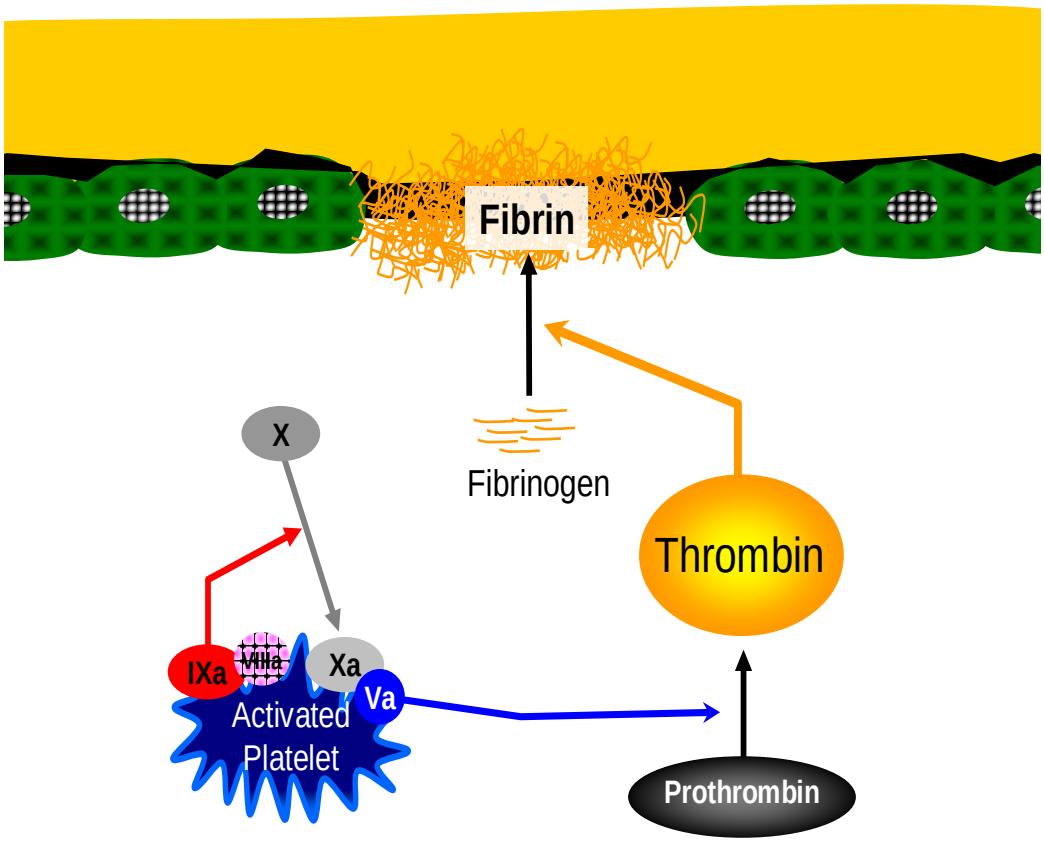


Figure 1.3. Fibrin strand formation

1.1.2. Fibrinolysis

To maintain blood flow and prevent venous occlusion soon after the clot is formed and the vessel repair is underway. The mechanism to dissolve the clot is initiated. This process is known as fibrinolysis.

During thrombus formation Tissue Plasminogen Activator (TPA) binds to polymerized Fibrin. This activates bound plasminogen molecules to produce Plasmin.

Through a systematic hydrolytic digestive process plasmin will digest polymerized fibrin strands to ultimately dissolve the clot and restore blood flow (Zorio *et al*., 2008).

Other proteins involved in the fibrinolytic pathway include: plasminogen, plasminogen activator inhibitor-1, tissue plasminogen activator, α_2-antiplasmin, urokinase, D-dimer and fibrin degradation products.

1.1.2.1. Plasminogen

Plasminogen is a 90 kDa protein made by the liver. It is a single chain protein with six structural domains, five kringle domains and a protease domain, each with different properties. During fibrin polymerization plasminogen binds to fibrin via the Kringle domains (Fig. 1.4).

Plasminogen activation requires plasminogen activators such as tissue plasminogen activator and urokinase plasminogen activator (t-PA, u-PA) which finally convert it into plasmin (Waisman 2002).

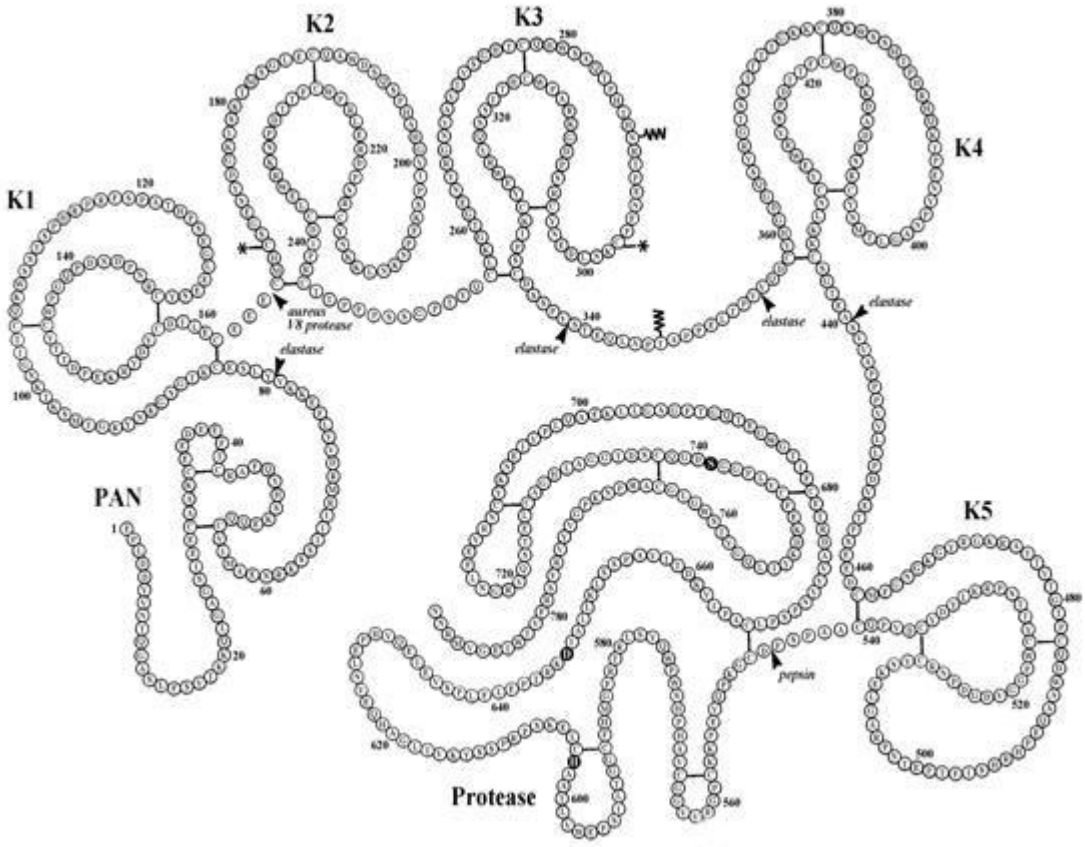


Figure 1.4. Plasminogen molecular structure (Picture taken from: http://www.chem.cmu.edu/groups/llinas/res/structure/hpk.html)

1.1.2.2. Tissue plasminogen activator (TPA)

TPA is the main activator of plasminogen. Made and secreted by the endothelial cells. TPA levels can increase with exercise, alcohol consumption or even drugs, like desmopressin acetate (DDAVP) (Girolami *et al*., 1995). Deep vein thrombosis risk is higher on people whose TPA levels do not increase with any of these stimuli. (Sue-Ling *et al*., 1987).

TPA binds to fibrin by covalent bonds on its two kringle regions. TPA occurs principally as a complex together with its main inhibitor PAI-1. Plasmin changes a single-chain TPA into a double-chain molecule. When a clot is formed the fibrin in it will help TPA with binding and orientation to be able to activate plasminogen which will eventually dissolve the thrombus.

1.1.2.3. Urokinase

Urokinase is a plasmin activator secreted by the urinary tract epithelial cells. It is present in small amounts in plasma and becomes attached to polymerized Fibrin, along with TPA at the time of clot formation. The molecule has only one single Kringle region which binds Fibrin.

1.1.2.4. Plasminogen activator inhibitor-1

The fibrinolytic process needs to be controlled by the body otherwise an increased destruction of fibrin could lead to big risk of bleeding. The main inhibitor of fibrinolysis is plasminogen activator inhibitor-1 (PAI-1) by stopping the action of TPA and/or urokinase. PAI-1 is made and secreted by the endothelial cells alongside with TPA.

1.1.2.5. α_2-antiplasmin

Plasmin, like Thrombin, is a potent enzyme and would be very destructive if free in the circulation. Therefore, the fast acting inhibitor, α_2-antiplasmin, binds to any free Plasmin localising its action to the site of the thrombus. It can also bind to Factors V and VIII and fibronectin. The α_2-antiplasmin can become crosslinked with Fibrin during polymerization, which makes Fibrin resistant to digestion by Plasmin.

1.1.2.6. Fibrin degradation products and D-dimer

When Plasmin binds to fibrin and cleaves the polypeptides the result are fragments known as fibrin degradation products (FDP).

There are several types of FDP called X, Y, D, E and D-D or D-dimer. Similar non-crosslinked breakdown products are also formed. Some of these fragments inhibit haemostasis by either inactivating platelets or preventing fibrinogen to polymerize into fibrin strands.

1.2. Platelet overview

Platelets are disc-shaped, non nucleated cells that are present in blood. They are involved in the cellular mechanisms of primary haemostasis leading to the formation of blood clots.

They are formed and extruded from a megakaryocyte (MK) cytoplasm in the bone marrow and released in to the circulation where their life-span is approximately 9 to 10 days. With a diameter of 1.5 to 3μm they are the smallest cells that circulate in peripheral blood. In health the reference range is 150 to 400 x 10^9/l.

The maturation process takes 4 to 5 days, where 2000 to 4000 platelets are released from every single Megakaryocyte (MK). Each day an adult produces 100 x 10^9 platelets which can increase 10 fold in times of higher demand.

The megakaryocyte-stimulating hormone, thrombopoietin (TPO), contributes to platelet count regulation via a primitive feedback loop. The majority of blood circulating platelets (20- 30%) are exchangeably sequestered within the spleen (splenic pooling) (Wilson *et al*., 2007).

1.2.1. Platelet structure and production

1.2.1.1. Internal structure

Platelets contain RNA, mitochondria, a canalicular system, a dense tubular system, cytoskeleton, several different types of granules, lysosomes, dense bodies and alpha granules (Fig. 1.5). The contents of the granules are released during platelet activation.

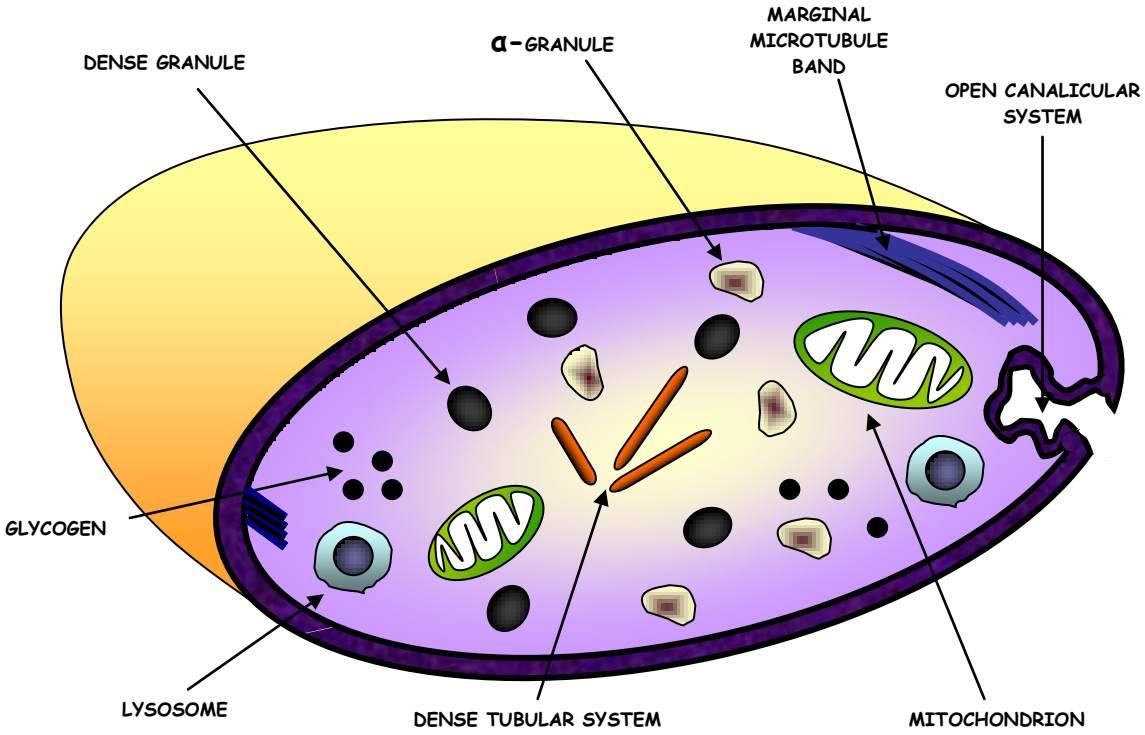


Figure 1.5. Platelet internal structure (Hoffbrand *et al*. 2001)

1.2.1.2. Granule contents

Platelets have several types of Granules:

-Alpha granules

-Dense granules

-Lysosomes

1.2.1.2.1. Alpha Granules

Alpha granules contain some coagulation factors, such as factor V, factor VIII and fibrinogen. The huge multimeric protein of von Willebrand Factor (involved in platelet adhesion and also a carrier for factor VIII in plasma), plasminogen activator inhibitor or PAI-1 (fibrinolytic inhibitor), α-antiplasmin, platelet derived growth factor and platelet factor 4 (neutralizes heparin effect) are also present in the platelet alpha granules.

1.2.1.2.2. Dense Granules

Dense granules contain some agonists for platelet activation, for example adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin (5-hydroxytryptamine or 5-HT) (involved in platelet aggregation), epinephrine (stimulates platelets to aggregate), and calcium (involved in platelet cytoskeleton functions and platelet shape change).

1.2.1.2.3. Lysosomes

Lysosomes are not involved in the platelet activation process. Lysosomes are cellular organelles that are involved in autophagy. Their contents are only involved in platelet metabolism and digestion. Amongst other molecules in the lysosomes, there are many digestive enzymes, like β-galactosidase, acid phosphate, α-mannosidase, β-glucosidase, β-*N*-acetylglucosaminidase and α-fucosidase .

1.2.2. Platelet functions

- Adhesion
- Activation and aggregation
- Procoagulant activity

Shear Stress in Flowing Blood

Inside the vessels, blood flow is governed by hydrodynamic shear stress forces (Morawietz *et al*., 2008). Blood velocity is maximal at the centre of the blood vessel and approaches zero at the vessel wall. The shear stress of a vessel also depends on the flow rate, the diameter of the vessel, and fluid viscosity. These are critical parameters in vascular cell adhesion.

The biggest blood cell types (e.g. white and red blood cells) tend to flow at the centre of the vessel leaving the vessel wall for the smaller cells, mainly platelets. This allows platelets to always be near a potential injury site.

1.2.2.1. Adhesion

The mechanism of haemostasis starts when vessel damage occurs. At the site of injury the endothelial wall is not longer intact and this will let the blood components to be in contact with subendothelial microfibrils which will start and trigger haemostasis. At this point, several molecules, such as von Willebrand factor and collagen are able to access their specific platelet receptors, glycoproteins GPIb-IX-V and GPIa / GPVI respectively (Fig. 1.6). von Willebrand factor and collagen binding to the platelet wall receptors leads to a quick increment of internal calcium ions from the platelet internal dense tubular sytem.

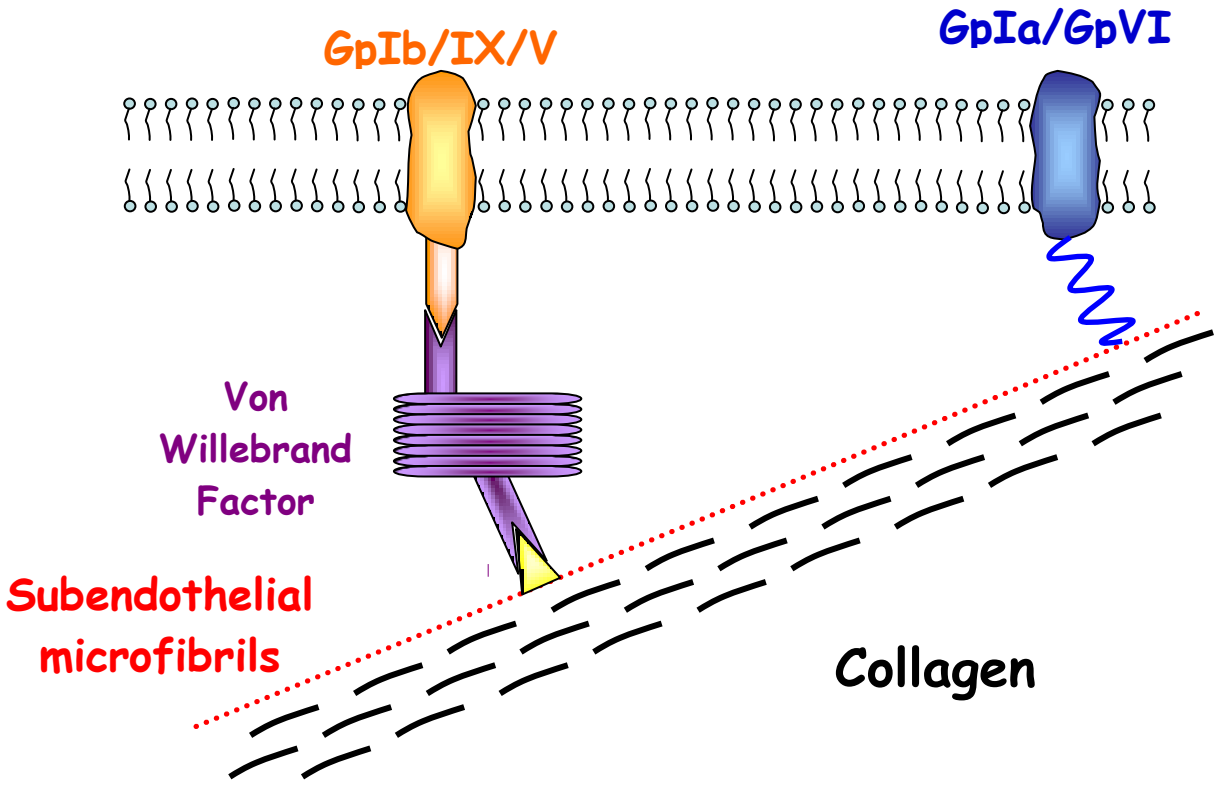


Figure 1.6. **Platelet Adhesion** (Hoffbrand *et al*. 2001)
Abbreviations: Glycoproteins (GPIb-IX-V and GPIa / GPVI)

1.2.2.2. Activation & aggregation

In normal circumstances, platelets remain in their original, inactivated state. This is in part achieved by the action of the endothelium. In an intact blood vessel, the endothelial cells release prostacyclin or prostaglandin I_2 (PGI_2) which inhibit platelet activation (Fig. 1.7).

When PGI_2 is released into the circulation it binds to platelet membrane receptors and this generates an internal signalling that increases the amount of cytosolic cyclic adenosine monophosphate (cAMP).

The direct consequence of increasing cAMP is that Calcium ions (Ca^{2+}) cannot be released from the dense tubular system (DTS), where they are stored. Ca^{2+} is needed for the platelet to go into a shape change. The actin and myosin fibers in the platelet cytoskeleton depend on Ca^{2+} to move.
Platelets that do not change shape also stay in a resting and inactivated state.

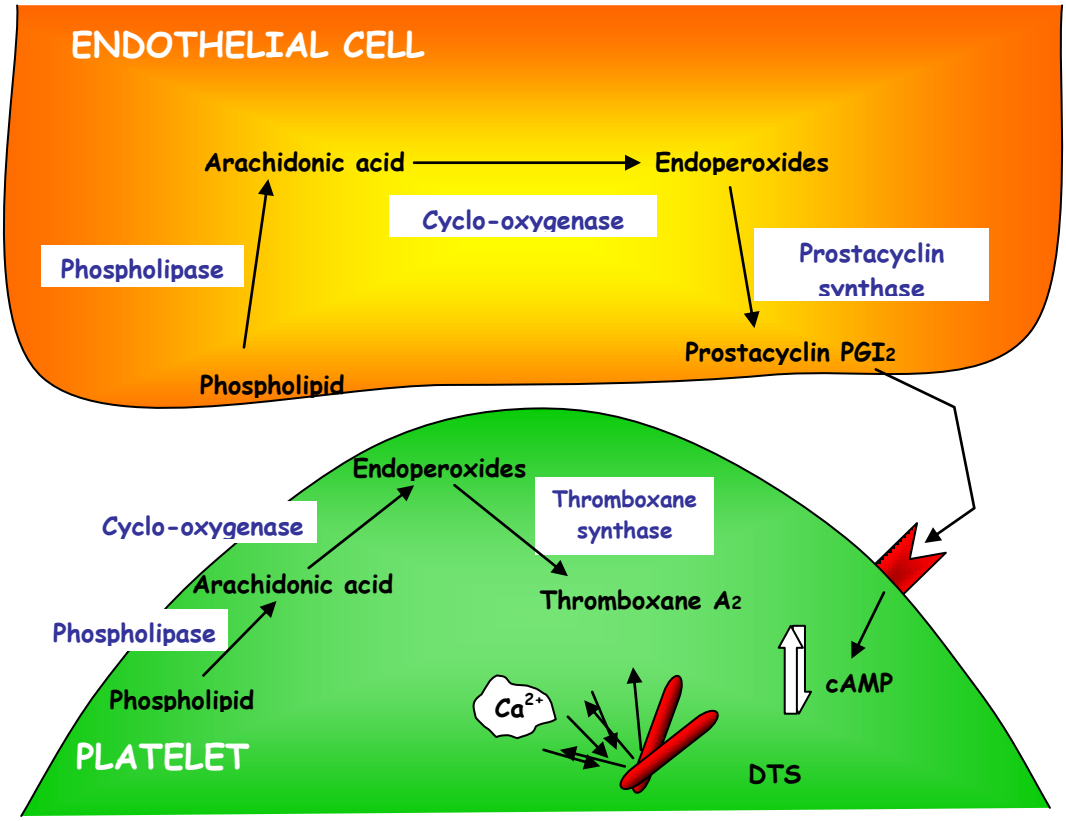


Figure 1.7. **Inactivated platelet.** (Hoffbrand *et al*. 2001)
Abbreviations: Calcium ions (Ca^{2+}), dense tubular system (DTS), cyclic adenosine monophosphate (cAMP).

When vessel damage occurs, platelets encounter a break in the endothelium where different molecules trigger their activation. The most common subendothelial molecule that activates platelets is collagen. There are few types of collagen that can do this, such as types I, III, and IV. (Saelman *et al*. 1994). Collagen can be found in any part of the human body but not inside a blood vessel. There are many more molecules and enzymes that can activate platelets, amongst these the most important are thrombin, thromboxane A_2 and ADP.

The process of activation involves several platelet changes:

Alpha and dense granules will release their contents by exocytosis. The release of Factor V, Factor VIII and fibrinogen triggers the coagulation pathway to generate more thrombin which will participate in the polymerisation of fibrinogen into fibrin. Secretion of these and other components will not only activate the platelet but will also attract more platelets to the site of injury and induce them to aggregate.

The production of thromboxane A_2 takes place in the platelet. Using the arachidonic acid pathway, the same pathway used by the endothelial cells to produce Prostacyclin. Thromboxane A_2 reduces the amount of cytosolic cAMP. Lower cAMP induces the release of Ca^{2+} into the platelet cytoplasm from the dense tubular system.
At this stage, the platelet is now ready to change its shape thanks to the extra Ca^{2+}. With the shape change, a new platelet receptor becomes active and available; this receptor is called GPIIb/IIIa and is necessary for platelets to aggregate, via fibrinogen binding. This platelet receptor can also be used by von Willebrand factor to adhere the platelet to the exposed subendothelium.

1.2.2.3. Procoagulant activity

Platelets third function is to provide with a phospholipid membrane that works as a procoagulant surface where the tenase and prothrombinase complexes take place and a site for thrombin generation.

To avoid excessive thrombin production on the surface of platelets, it is necessary activated protein C (APC) and protein S (cofactor) which will stop FVIII and FV to become active and therefore interfere with the coagulation cascade. Lack of APC does not stop protein S from lowering thrombin generation (Sere *et al*., 2004).

On the other hand, patients who have low protein C and S levels have a higher risk of thrombosis. Factor V Leiden, prothrombin G20210A mutations also give a much higher risk of developing a clot. (Young *et al*, 2003).

1.3. Critically Ill patients

1.3.1. Intensive Care Unit (ICU)

An intensive care unit (ICU) is the area of a hospital that treats and monitors trauma or critically ill patients.

Patients that end up in this department can come from the accident and emergency department, from a different ward or even straight from surgery. The ICU is a high technology, highly specialized and very expensive department.

Not all ICUs are the same; some are more specialized than others, depending on the type of hospital they are in. Some just deal with severe injury or trauma. Many can be part of units treating for specific conditions such as heart, liver, kidney or neurological diseases. Others can specialise on a specific age group (neonatal).

Haematological

Coagulation disorders and acquired coagulopathies are often seen in the ICU. The most common problems associated with haemostasis are liver disease, disseminated intravascular coagulation, anti-coagulant drugs, vitamin K deficiency and blood transfusions.

First line of investigation in the ICU is to perform a clotting screen, which includes PT and APTT. They also can measure the thrombin time, fibrinogen and D-dimer levels.

1.3.2. APACHE scoring system

The APACHE scoring system (Acute Physiology, Age, and Chronic Health Evaluation) is the gold standard tool in the ICU to accurately describe a patient's status and stability and to measure the risk of mortality once admitted in the hospital.

APACHE has been updated 3 times. The original APACHE was developed in 1981 at the George Washington University Medical Centre as a way to measure disease severity and it used 34 different parameters (Knaus *et al.*, 1981). APACHE II (Knaus *et al.*, 1985) is a much more simplified version of the first APACHE; it only uses 14 physiological variables:

1. Temperature - core
2. Mean arterial pressure
3. Heart rate
4. Respiratory rate
5. Oxygenation
6. Fraction of inspired oxygen (FIO_2)> 0.5 alveolar-arterial oxygen tension difference (A-aDO_2)
7. FIO_2 < 0.5 partial oxygen arterial pressure (PaO_2)
8. Arterial pH
9. Serum sodium concentration

10. Serum potassium concentration
11. Serum creatinine concentration
12. Haematocrit
13. White blood cell count
14. Glasgow coma score

APACHE III (1991) uses data from 40 hospitals and more than 17,000 patients (Wong *et al*., 1991). New variables were added to the APACHE III:

15. Prior treatment location
16. Disease requiring ICU admission

APACHE III also takes into account patient's age and chronic as part of the scoring system. The final score will give a numerical value for predicted mortality. APACHE III is part of a very clever system which gets updates on almost a daily basis.

1.4. Acidosis

Critically ill patients often develop some degree of acidosis. Acidosis is described as any pH lower than 7.35 or physiological pH. For humans pH is measured as arterial pH; one of the most common ways of testing it is by doing an arterial blood gas analysis. It is also necessary to look for the underlying condition that can be causing the low pH. Acidosis is a serious complication of gravelly ill patients and therefore needs to be treated as an emergency.

Physiological pH can fluctuate slightly between 7.35 and 7.45. Anything lower than pH 6.8 or higher than pH 7.8 in humans will be incompatible with life. Irreversible damage can be caused to the cells and tissues at these extreme pH levels (Needham, 2004).

Acidosis is chemically speaking, a higher concentration of H+ in blood than in normal conditions. Diseases like diabetes or simply starvation or even a bad diet can lead to lower body pH.

1.5. Hypothermia

Hypothermia in humans is defined as any body temperature lower than 37°C. Hypothermia is often seen in patients admitted in the ICU. Hypothermia is classified in three different stages (McCullough *et al*., 2004).

Stage 1: Comprises body temperature between 36°C to 35°C, patient starts shaking and loses hand ability to perform precision tasks, numbness also appears. Breathing is fast and shallow. Blood supply to arms and legs is lowered to avoid body temperature to fall even more, this is achieved by vasoconstriction.

Stage 2: at this stage body temperature has gone down to 35°C to 33°C, now the shaking has turned into strong shivering. Now the patient has lost most of his coordination, moving slowly and with difficulty. Vasoconstriction is higher now and the blood supply to arms and legs is almost zero because the body is trying to keep the warm blood to the vital organs. Now the patient's skin is completely pale with even blue lips and ears.

Stage 3: Anything below 32°C is considered as very dangerous temperature and hypothermia reaches its maximum level of severity. The patient now has lost his ability to walk or even to think properly or to be aware of what is happening. Blue pale skin is observed all around the body. Breathing rate slows down and heart starts racing. At this stage important organs start to shut down and the victim will soon pass away.

1.5.1. Temperature

There are two main types of body temperature that can be measured. First, is the external or the also called peripheral temperature; this will drop first to avoid unnecessary lose of heat that is needed to keep vital organs warm. The second type is called core temperature; this is the important which needs to be kept inside the normal physiological range to avoid any further complications.

1.6. CLOTTING SCREENS

1.6.1. History

Screening tests provide an assessment of the 'extrinsic' and 'intrinsic' systems of blood coagulation and also the central conversion of fibrinogen to fibrin.

1.6.2. Prothrombin Time

With this assay the extrinsic coagulation pathway is timed. The factors directly involved are Prothrombin, fibrinogen and factors V, VII and X. PT assay only uses to main reagents which will be added to plasma obtained from citrated blood. These two main reagents are Thromboplastin and calcium. Normal reference PT times are between 10 and 14 seconds. (Hoffbrand *et al*., 2001). Any time delay on the PT will lead to further investigations on the factor levels involved.

1.6.3. Activated Partial Thromboplastin Time

This assay measures the also called intrinsic pathway. Many more coagulation factors are involved in the APTT, such as Prothrombin, fibrinogen and Factors VIII, IX, X, XI and XII. To perform an APTT three main reagents

are needed: firstly, something to trigger contact activation such as kaolin or silica; secondly, basic blood components need to be added to the plasma, these are calcium and phospholipids. Normal reference APTT times are between 30 to 40 seconds. (Hoffbrand *et al*., 2001).

1.6.4. Stago Start4® Analyser

The STart® 4 is a coagulation analyzer that can be used to measure Prothrombin time, Activated partial thromboplastin time and thrombin time. It has a clot based detection system but with some modifications applied by Diagnostica Stago Company. This method is called Viscosity-based Detection System®. This technology allows the operator to run any plasma sample without having to discard the ones that have some degree of turbidity, such as samples with excess fat or lipaemic, etc. This is a big advantage compared to other optical clot detection methods. The STart® 4 analyser can run up to 4 samples simultaneously at 4 independent cuvette locations.

The system uses a steel ball inside a cuvette, the ball moves thanks to an electromagnetic field generated by to coils working in turns. When reagents are added to the plasma, time starts counting and the ball moves from side to side until clot formation. This will make the plasma so viscous that the ball cannot longer move. This will stop the clock. The machine finally, shows the clotting time on the screen (Fig. 1.8).

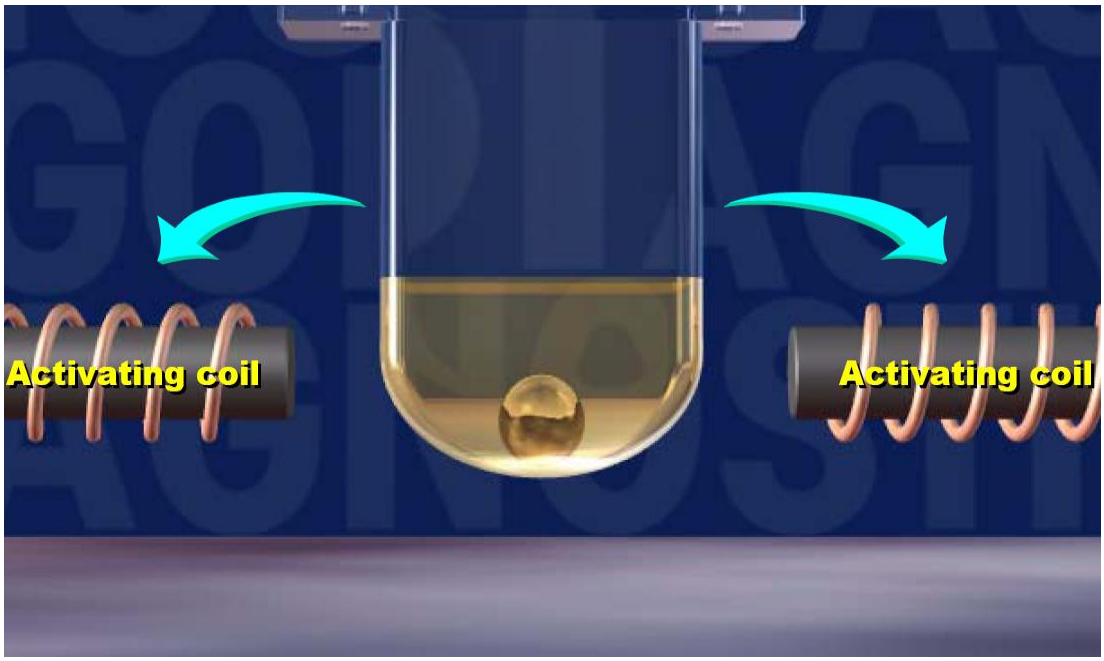


Figure 1.8. Viscosity-based detection system
(http://stago-us.com/images/vds.pdf)

The temperature of the cuvette can be adjusted down to 31°C. APTT and PT were performed on this machine at different temperatures and different pHs.

1.7. GLOBAL ASSAYS

1.7.1. Calibrated Automated Thrombography (CAT)

1.7.1.1. Principle of the Technique

Calibrated Automated Thrombography (CAT) is a method that allows measuring Thrombin generation. The assay can be run on Platelet Poor Plasma (PPP) or Platelet Rich Plasma (PRP).

The CAT uses a thrombin specific substrate that is added in excess. It is a fluorogenic substrate and once bound to thrombin emits fluorescence at specific wave length that is detected and registered by computer software.

Samples are run in two parallel assays at the same time. The first assay runs the sample with the reagents to trigger the coagulation system; the second assay runs the sample plus a calibrator, this will allow the machine to accurately calibrate the specific optical properties of any given plasma (i.e. optical density).
Each patient's plasma absorbs light differently depending on the amount and types of molecules present in it. This is called "inner filter effect" (Hemker et al., 2002).

The CAT software converts substrate + thrombin fluorescence to amount of thrombin. Once the assay is finished, the computer shows a graph with two axes. Amount of fluorescence will be expressed on the Y-axis and time on the X-axis; this is the raw curve obtained from a direct measurement of fluorescence. The amount of thrombin is proportional to the amount of fluorescence.

The software makes a first derivative from the raw curve and the result is a bell shaped curve known as a "Thrombogram" (Fig. 1.9). On the Thrombogram the amount of thrombin (Y axis) is plotted against the time (X-axis) (Hemker et al., 2002).
The thrombogram has several parameters: Lag time, peak, endogenous thrombin potential (ETP), time-to-peak and tail.

1.7.1.2. Lag Time

Lag time is the time it takes for the process of coagulation to begin, i.e. the first burst of thrombin production (Normally, around 10nmol of thrombin are formed). It is the first flat part of the thrombogram graph. It starts when the last reagent is added to the measurement well; in this case, $CaCl_2$ and the thrombin substrate. The lag time is the equivalent of a clotting screen (APTT or PT) and therefore the lag time finishes when the clot starts to form.

1.7.1.3. Peak

It is the maximum concentration of thrombin formed per unit time.

1.7.1.4. Time to Peak

It is the time that the sample takes to reach the peak of the thrombogram.

1.7.1.5. Endogenous Thrombin Potential

The Endogenous Thrombin Potential or ETP is the total amount of thrombin that a given sample can generate. It is represented in the Thrombogram as the area under the curve (AUC).

1.7.1.6. Tail

The tail of the thrombogram is the time at which the thrombin concentration comes back to zero.
Alpha 2-macroglobulin is one the main inhibitors of thrombin. The substrate used in the CAT also binds to Alpha 2-macroglobulin + thrombin. The CAT software automatically subtracts that fluorescence from the total and this is the reason that the tail can reach the baseline.

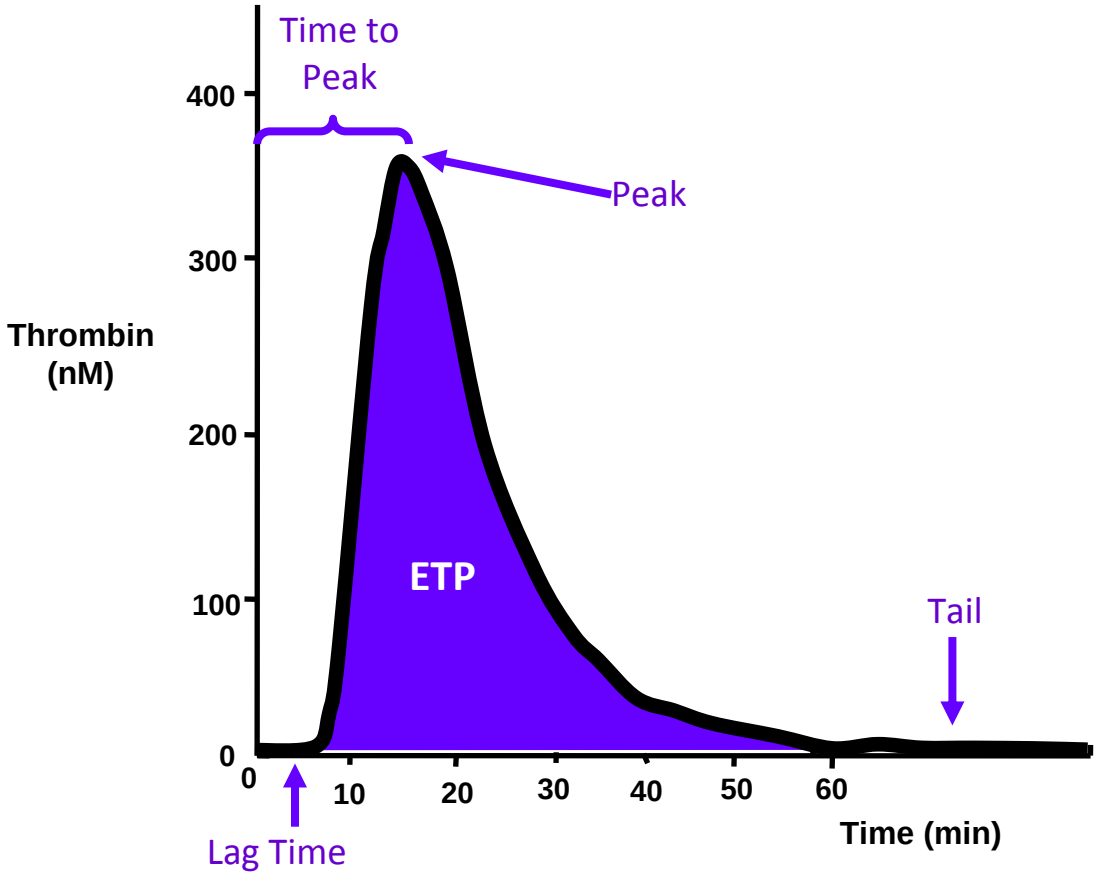


Figure 1.9. Calibrated Automated Thrombogram
Picture based on: http://www.haematologica.it/cgi/content/full/92/12/1639/F1921639

1.7.2. Thromboelastometry (TEG)

1.7.2.1. History

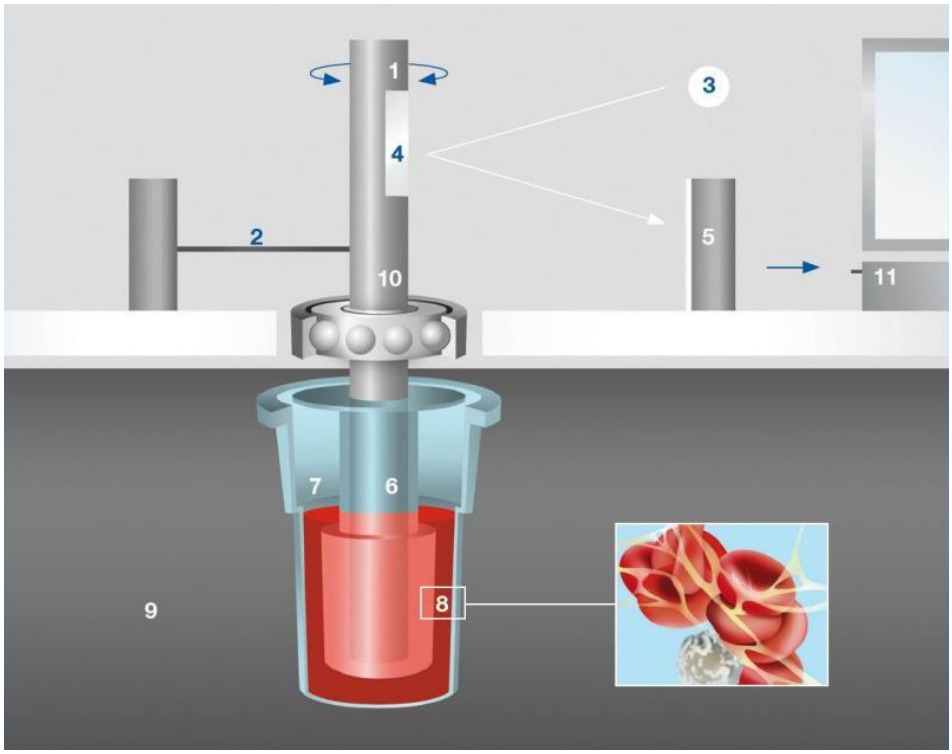
Thromboelastography (TEG) was developed and described by Dr. Hellmut Hartert back in 1948. This assay looks at clot formation, time to clot, clot stability, clot strength and also the fibrinolytic pathway.

The TEG is considered part of the global assays family, where the whole haemostatic process can be measured with one single test. One of the main

advantages of Thromboelastography is that it can be run even on whole blood. This means that all the blood components and red cells that are removed normally with spinning are still going to take part in the measurement. (Luddington *et al*., 2005).

The ROTEM® or rotational thromboelastometry is frequently used during several types of surgery (such as cardiac surgery and liver transplantation) to monitor patient haemostatic status. The ROTEM® assay can give information to surgeons and doctors in no more than 30 minutes. Also the analyser is so compact and so simple to run that it can even be taken into the operation room. The ROTEM® has also been used in the ICU and where is has demonstrated it is a very good predictor any transfusion requirements (Kaufmann *et al*., 1997).

Thromboelastometry has also many applications and advantages in the laboratory. Because it is a global assay is very useful In areas such as thrombophilia screening and prediction of thrombosis (Traverso *et al*., 1993; O'Donnell *et al*., 2004).
The haemophilia clinic also benefits from the use of the ROTEM®. Is very good monitoring treatments where recombinant factors are needed (Hayashi *et al*., 2004; Sorensen & Ingerslev, 2004).



1 oscillating axis
2 counterforce spring
3 light beam from LED
4 mirror
5 detector (electr. camera)
6 sensor pin
7 cuvette with blood sample
8 fibrin strands & platelet aggregates
9 heated cuvette holder
10 ball bearing
11 data processing unit

Figure 1.10. ROTEM® Instrument (http://www.rotem.de/site/index.php?option=com_content&view=article&id=1&Itemid=7&lang=en)

1.7.2.2. Method

The sample is placed inside a single-use cuvette in a warm cuvette holder. The detection sensor will be submerged into the sample that is inside the cuvette. This sensor is also attached to a rotating axis that turns 4.75° to the left and 4.75° to the right alternatively.

The analyser measures elasticity with the help of a spring attached to the rotating axis. The axis has a little mirror that serves to be located by a computer detector. Once clot formation occurs, a change in the viscosity and elasticity affects the movement of the rotating axis. Finally, this is registered by the computer which converts the signal into a thromboelastogram.

1.7.2.3. Parameters

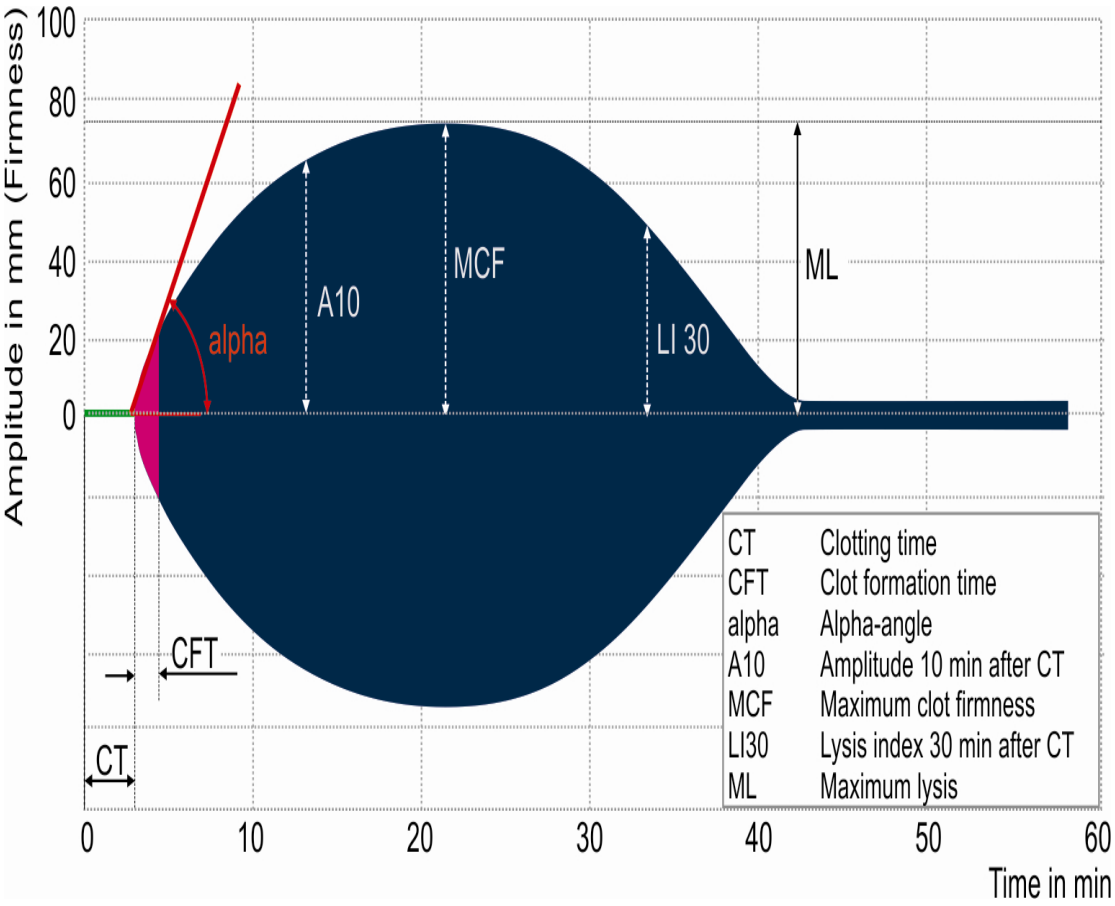


Figure 1.11. Thromboelastogram Parameters (http://www.rotem.de/site/index.php?option=com_content&view=article&id=2&Itemid=8&lang=en)

1.7.2.3.1. Clotting time

Clotting time (CT) is the time taken from the start of measurement until initiation of clotting (Fig. 1.11). It is a very useful parameter that helps on controlling the use of heparins in the operation room (Bowers *et al*., 1993).

1.7.2.3.2. Clot firmness

Clot firmness is defined as the amplitude (mm) of thromboelastogram curve at any given time.

1.7.2.3.3. Clot formation time

Clot Formation Time (CFT) is the time (sec) taken from initiation of clotting until the width of a clot firmness of 20mm is detected. (Fig. 1.11).

1.7.2.3.4. Maximum clot firmness

Maximum Clot Firmness (MCF) is the maximum width or amplitude (mm) of the thromboelastogram curve (Fig. 1.11).

1.7.2.3.5. Maximum lysis

Maximum lysis (ML) is the reduction (%) of the clot firmness after MCF in relation to MCF. Fibrinolysis is defined when ML is bigger than 15% within 1hour (Fig. 1.11).

1.7.2.4. Tests types

1.7.2.4.1. EXTEM

EXTEM as its name suggests is an assay that looks at the extrinsic pathway of the coagulation cascade. To initiate this test human tissue factor (TF) is added (as part of the EXTEM reagent) to the plasma sample.

Only coagulation factors that are involved in the extrinsic pathway are the ones responsible for the results of this test. Fibrinogen and platelet defects can also affect the EXTEM results.

The EXTEM reagent has some type of heparinase that deals with any heparin contamination.

1.7.2.4.2. INTEM

INTEM measures the intrinsic pathway of the coagulation cascade. INTEM reagent has some molecules that will initiate the coagulation cascade by contact activation. All intrinsic coagulation factors can affect this assay, such as prothrombin, fibrinogen and factors VIII, IX, X, XI and XII.
Heparin contamination prolongs clotting times and alters all results obtained by INTEM.

1.7.2.4.3. NATEM

This is the simplest test run on the ROTEM®. Only calcium is added to the sample to initiate the haemostatic system.

1.8. CONTACT INHIBITOR

Corn trypsin inhibitor (CTI) is a small protein that is localized in the kernels of most species of corn. CTI is not only an inhibitor of trypsin, but is also a specific inhibitor of human factor XIIa. CTI binds to factor XIIa. This will result in an extension of the APTT when testing plasma + CTI but the PT will remain unaffected. CTI is obtained from sweet corn and purified by chromatography. (http://www.haemtech.com/Inhibitors_Substrates/CTI.htm).

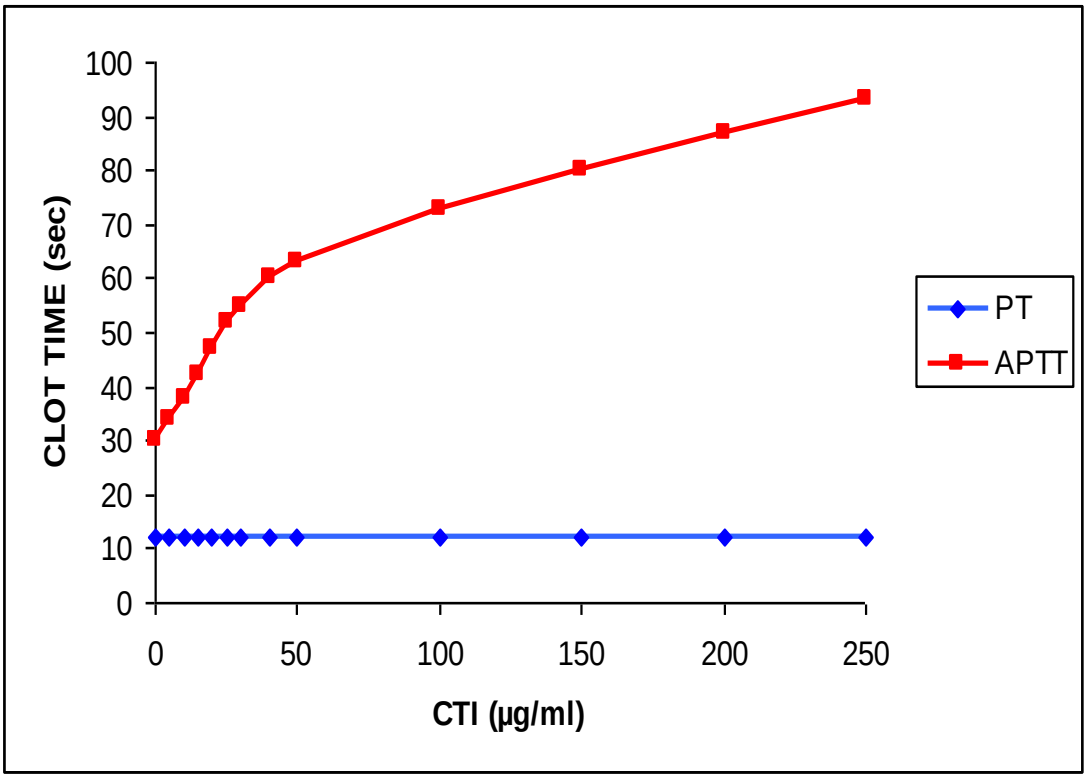


Figure 1.12. Effect of CTI on the APTT and PT of normal human plasma

(http://www.haemtech.com/Inhibitors_Substrates/CTI.htm)

Abbreviations: Corn Trypsin Inhibitor (CTI), Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT).

The Figure illustrates how CTI affects the APTT and PT of normal plasma (Figure 1.12.).

The use of Corn Trypsin Inhibitor for the measurement of thrombin generation by calibrated automated thrombography (CAT).

Contact factor-activated thrombin generation is completely inhibited at a CTI concentration of 18.3 µg/ml whole blood. Anticoagulation of blood is achieved by inhibition of the contact pathway by CTI (Luddington *et al*, 2004). Increasing the CTI concentration above this level did not lead to suppression of the TF-triggered ETP.

1.9.THESIS AIMS

-To study the effects of acidosis and/or hypothermia on the haemostatic process.

-To establish reference physiological ranges of coagulation parameters under acidosis and hypothermia.

-To develop a better protocol to monitor haemostasis on trauma and critically ill patients in the Intensive care unit.

-To compare standard coagulation procedures with more advanced Assays, such as the Calibrated Automated Trombography and Rotational Thromboelastometry.

2. Materials and methods

2.1. Samples

2.1.1. Method of bleeding

Blood was collected from 40 consenting healthy adults and also from 17 critically ill patients in the Intensive Care Unit at Addenbrooke's Hospital, NHS Trust. Cambridge, UK. Ethical permission was obtained to collect the samples (Apendix).

2.1.2. Tubes

Sample tubes used were S-monovette® 9NC (PFA) with a total capacity of 3.8ml containing 0.129 mol/l of trisodium citrate and citric acid buffer solution pH 5.5 (0.38 ml citrate buffer solution) (Sarstedt Ltd, Leicester, UK).

These tubes were chosen because they contained a buffer which would preserve the original pH of the sample.

2.1.2.1. Corn Trypsin Inhibitor

Corn Trypsin Inhibitor (12.66 µl CTI) (Haematology Technologies Inc., VT, USA) was added to each tube before use, giving a final concentration of 1.45µmol/L of CTI.

2.1.2.2. Buffers

-Working buffer: (20mM Hepes, 140mM NaCl, 5 mg/ml BSA)

-Fluo-Buffer: [875µl buffer (20mM HEPES, 60mg/ml BSA), 300µl 1M $CaCl_2$ and 75µl of fluorogenic substrate Z-Gly-Gly-Arg-AMC (diluted with 100mM DMSO)]

2.1.3. Processing & storage

2.1.3.1. Platelet poor plasma

To obtain platelet poor plasma (PPP) from whole blood, samples were centrifuged at 4000rpm (3578g) for 15 minutes. The plasma was separated into a 2ml Eppendorf tube using a Pasteur pipette.

2.1.3.2. Platelet rich plasma

To obtain platelet rich plasma (PRP) from whole blood, samples were centrifuged at 800 rpm (143g) for 8 minutes. The plasma was separated into a 2ml Eppendorf tube using a Pasteur pipette.

To be able to standardize the PRP, a final count of 150 x 10^9/L platelets was used for each PRP sample. To calculate this, the Beckman Coulter® LH750 (Beckman Coulter, Miami, USA) was used to estimate the platelet number of a given sample, then the PRP was diluted proportionally with PPP from the same sample, to finally obtain the required 150 x 10^9/L platelet count.

PPP samples were kept on ice for immediate use or frozen down at -80°C for later use. PRP samples were run within 6 hours and had to be kept at room temperature to avoid platelet activation/damage.

When PRP was diluted with PPP, PPP had to be at room temperature before mixing to avoid a thermal shock which could affect the status of the platelets.

2.1.4. pH manipulation

For the pH measurement of different samples a pH 210, Microprocessor pH Meter (Hanna Instruments, Ltd.) was used. This instrument has a sensor probe made of glass, which needs to be submerged into the sample tube to give a pH reading.

pH was always measured at room temperature. A duplicate sample was used to avoid possible contact activation with the glass probe of the pH meter.
For correction or change of sample pH, sodium hydroxide (15.2M), AnalaR®, BDH, (VWR International Ltd.) or lactic acid (11.6M), AnalaR®, BDH, (VWR International Ltd.) were used.

Both were diluted to 1:10 with deionized water because the reagents were too strong for the volume of plasma obtained from patient samples.

Reagents were also pH manipulated with NaOH and/or lactic acid, to match the target pH of the experiment.

Two different pH values were used for this study: a physiologic pH at 7.35 and an acidotic pH at 6.9, to try to imitate the potential acidosis found in ICU patients.

2.2. Automated assays

2.2.1. The Calibrated Automated Thrombogram (CAT)

A Fluoroskan Ascent® FL (Thermo Fisher Scientific Inc.) was used to run the CAT and the software used for this assay was Thrombinoscope^{TM} Software version 3.0.0.25. (Thrombinoscope BV, Maastricht, The Netherlands).

2.2.1.1. Principle

The phospholipid vesicles were prepared as described by Falls *et al.* (2000). The lipids were combined in the required proportions and dried under nitrogen at 45°C for 30 minutes and then resuspended in HEPES buffer (50mM HEPES, 145 mM NaCl, pH 7.1). The mixture was then passed through an extrusion device 11 times using a 100nm diameter polycarbonate filter. A further 21 passes were then performed through a 50nm diameter polycarbonate filter and the resultant vesicles were stored at 4°C until use.

Test principle:

The assay was performed as described by Hemker *et al.*, 2003. Generally speaking, the machine runs two parallel assays for each sample; the first assay uses 20µl of thrombin calibrator and 80µl of sample plasma. This is used to correct the internal filter effect by calibrating the colour of any given plasma.

The second assay uses 10µl of 4µM of phospholipids vesicles, 10µl of 1.5pM of recombinant human tissue factor and 80µl of sample plasma.

When the microplate is inserted into the Fluoroskan, 20µl of calcium chloride and the fluorogenic substrate, which have been mixed previously [875µl buffer (20mM HEPES, 60mg/ml BSA), 300µl 1M $CaCl_2$ and 75µl of fluorogenic substrate (diluted with 100mM DMSO)] were added by an automated dispenser to all the wells in use. With a final volume of 120µl the reaction initiated and was monitored by Thrombinoscope software.

2.2.1.2. Reagents

-Thrombin Calibrator, Thrombinoscope BV, Maastricht, The Netherlands.

-Recombinant human tissue-factor, Dade® Innovin®, Dade Behring, Germany.

-Phospholipid Vesicles (see 2.4.1 preparation of phospholipid vesicles)

-Fluorogenic Substrate Z-Gly-Gly-Arg-AMC (Bachem, Dubendorf, Switzerland)

-Calcium chloride (BDH Ltd, Poole,UK)

-HEPES (BDH Ltd, Poole,UK)

-Bovine Serum Albumin (BSA)(Sigma Aldrich Ltd, Poole UK)

-Dimethyl sulphoxide (DMSO) (Sigma Aldrich Ltd, Poole UK)

Preparation of Phospholipid vesicles:

Instrumentation and consumables:

Extrusion device (Avestin, Ottawa, Canada)

Polycarbonate filters (Glen Creston, Middlesex, UK)

Reagents:

Synthetic phospholipids in chloroform (Avanti Polar Lipids, Alabama, USA): phosphatidyl-choline (PC), phosphatidyl-ethanolamine (PE), phosphatidyl-serine (PS).

2.2.1.3. Controls

Plasma reference control was used in all rounds and it was obtained from healthy individuals.

2.2.1.4. Calibrators

Thrombin calibrator was used as described in Section 2.2.1.2.

2.2.1.5. Plates

The Fluoroskan Ascent® FL uses 96 well microplates, Blue, U-shape (Greiner Bio-One GmbH, Germany). This plates allows to run up to 24 samples at a time (each sample needs 2 wells with the calibrator + 2 wells with the sample).

2.2.1.6. Temperature adjustment

The temperature was set through the Thrombinoscope software.

By the time the reaction starts the microplate and all of the assay components in the wells are already at the required temperature. The incubator temperature range is from room temperature +3°C to +45°C, when ambient temperature was at 25°C.

2.2.1.7. pH adjustment

All reagents and plasma samples were adjusted with lactic acid (1.16M) and/or sodium hidroxide (1.52M) to obtain the pH value required, before commencing the reaction.

2.2.1.8. The measurement

Thrombinoscope software monitors the reaction by detecting the fluorescence emitted by the cleaved fluorogenic substrate. The filters on the Fluoroskan have an excitation wavelength from 320nm to 700nm and an emission wavelength range from 360nm to 670nm. The signal is monitored on intervals of 30 seconds during the whole length of the assay.

Thrombinoscope ^{TM} converts the direct reading of emitted fluorescence into units of thrombin (mmol) to produce a raw curve. A first derivative is obtained from the raw curve, giving a classic bell shaped curve where parameters such as: Lag time, peak and time-to-peak can be derived (Hemker *et al.*, 2003).

Thrombinoscope ^{TM} also subtracts the α_2-macroglobulin bound thrombin contamination from the bell shaped curve, bringing it back to baseline; therefore creating a curve tail and delimiting the area under the curve or endogenous thrombin potential (ETP).

2.2.2. The TEG

The system is called rotational thromboelastometry (ROTEM®) (Pentapharm Gmbh, Munich, Germany), with a four channel measurement unit and an electronic pipette in station. The software is already incorporated in the analyzer.

2.2.2.1. Principle

The ROTEM can run five different types of assays. The one used for this study was the EXTEM Assay. This is a fast assessment of clot formation, fibrin polymerisation and fibrinolysis via Tissue factor activation. It uses 20µl of Star-TEM®20, 20µl of Ex-TEM® and 300µl of plasma (PPP/PRP).

2.2.2.2. Reagents

-Star-TEM®20 (0.2 mol/l of $CaCl_2$ in HEPES buffer pH7.4 and 0.1% NaOH) (Pentapharm Gmbh, Munich, Germany).

-Disposable cups and pins (Pentapharm Gmbh, Munich, Germany).

2.2.2.3. Sample preparation

Whole blood samples extracted using a citrated tube (S-Monovette 3.8ml 9NC PFA).

PRP and PPP samples were obtained from whole blood as described in Sections 2.1.3.1 and 2.1.3.2.

2.2.2.4. Controls

No control samples were required for this assay.

2.2.2.5. Temperature adjustment

The temperature was set before running the analysis, through the software menus. The temperature range of the analyzer was between 32°C and 39°C.

2.2.2.6. pH adjustment

All reagents and plasmas were adjusted with lactic acid (1.16M) and/or 1:10 sodium hidroxide (1.52M) to obtained the pH value required, before commencing the reaction.

2.2.2.7. The measurement

Blood was added to a disposable cuvette in a heated cuvette holder. A disposable pin (sensor) was fixed on tip of the rotating axis and this axis is guided by a high precision ball bearing system.

The axis rotates back and forth and is connected with a spring for measurement of elasticity. The exact position of the axis is detected by reflection of light on small mirror on axis. The loss of the elasticity when the sample clots, leads to a change in the rotation of the axis.

Once the data is obtained, are then analysed on a computer and visualised in a thromboelastogram.

2.2.3. The START4

Manual APTT and PT (STart® 4). The STart® 4 (Stago Diagnostica Ltd., USA) semi-automated haemostasis analyzer features the method of Electro-mechanical Clot Detection® (Viscosity-based Detection System). The system uses a steel ball inside a cuvette, the ball moves thanks to an electromagnetic field generated by to coils working in turns. When reagents are added to the plasma, time starts counting and the ball moves from side to side until clot formation. This will make the plasma so viscous that the ball cannot longer move. This will stop the clock. The machine finally, shows the clotting time on the screen.

2.2.3.1. Prothrombin time

PT reagents:

Human Thromboplastin, Simplastin® HTF (BioMerieux Inc., USA)

PT Assay: plasma 100µl was added to a cuvette and incubated at the set temperature for 1 minute. After this, 200µl of Simplastin® HTF was added and the reaction initiated.

2.2.3.2. Activated partial Thromboplastin time

APTT reagents:

- Micronized-Silica [0.175g of Fumed Silica 99.8%; Sigma-Aldrich Chemie Gmbh., Germany in 100ml of MDA® Imidazole buffer (Diluent) (Trinity Biotech, USA)].
- Platelet substitute, Diagen® (Diagnostic Reagents Ltd., Oxon, UK)
- 0.025M Calcium Chloride (BioMerieux Inc., Durham, UK).

APTT Assay: platelet substitute (50µl), 50µl of micronised–silica and 50µl of plasma were incubated for 4 minutes. To initiate the coagulation cascade, 50µl of calcium chloride are added.

2.2.3.3. Sample preparation (PPP, PRP)

PPP and PRP were obtained as described before in Sections 2.1.3.1. and 2.1.3.2.

2.2.3.4. Temperature adjustment

The assay temperature of the Start®4 was set in the range 31°C to 40°C. The current temperature of the analyzer was constantly shown on the liquid crystal screen.

2.2.3.5. pH adjustment

All reagents and plasma samples were adjusted with lactic acid (1.16M) and/or sodium hydroxide (1.52M) to obtain the pH value required, before commencing the reaction.

2.2.3.6. The measurement

PT or APTT Assays were selected from the menu. The attached automated pipette has become active and ready to load the reagents and the plasma into the cuvette containing a ball bearing in it. The reaction began and the timer started counting until the clot was so solid that the ball bearing could not move. The time taken was noted by the machine software.

2.3. Statistical software

SPSS Statistics (PASW Statistics 18) software was used (www.spss.com/statistics/).

Data obtained from this study needs to be distributed in several groups according to the different parameters used (mainly temperature and pH) and the different assays used (PT, APTT, CAT and ROTEM). When comparing two or more groups of data, is not possible to use a t-test comparison because of the problem of multiple testing. The best option is to use a one-way analysis of variance (**ANOVA**). With ANOVA it is possible to find out if the means of the populations differ.

When ANOVA leads to a conclusion that there is evidence that the group means differ, it is necessary to investigate which of the means are different. This is where the **Tukey** multiple comparison test is used. The test compares the difference between each pair of means with appropriate adjustment for the multiple testing. The results are presented as a matrix showing the result for each pair, as a P-value, **p values of <0.05 were considered significant**. The Tukey multiple comparison test, like ANOVA, assumes that the data from the different groups come from populations where the observations have a normal distribution and the standard deviation is the same for each group.

T-test for Equality of Means was also used for simpler comparisons between two different groups of data.

3. PROBLEM SOLVING

3.1. pH measurement & sample activation.

It quickly became apparent that, somehow, the pH manipulated samples were activated. Consequently, the whole pH manipulation process was analyzed and investigated.

A pH 210, Microprocessor pH Meter (Hanna Instruments, Ltd.) was used to measure sample pH. This pH meter has a probe which needs to be submerged into the sample. The probe is made of glass and this could be a problem. Glass is negatively charged and behaves as a potent solid phase activator of the contact activation process (Sánchez *et al*, 2002). When plasma comes in contact with glass, FXII becomes activated as the βFXIIa form and therefore the coagulation cascade is initiated.

Importantly, the samples used for this study had CTI which limits endogenous sample contact activation (Luddington *et al*, 2004). CTI is not only specific for trypsin but it is also capable of inhibiting FXIIa from the plasma. With no FXIIa coagulation can not be initiated.

The concentration of CTI used is sufficient to prevent the endogenous contact activation associated with sample collection and separation. However, it is not sufficient to prevent the additional challenge of contact with a glass surface as presented in the pH probe. This effect can be demonstrated by using the ROTEM®. When a CTI treated whole blood sample is run on the analyzer, the graph obtained should be a completely flat line following adding the calcium. However, eventually the CTI becomes overwhelmed by the contact activation surface of the ROTEM® cuvette and following a prolonged lagtime a normal trace is generated.

3.2. pH measurement design.

To investigate the possibility that the pH meter probe activated the sample, ten samples were run in duplicate. Half of the duplicate samples had glass probe contact for a minute while the other half did not have any contact. All the duplicates were run with the CAT under the same conditions. Results are given on Table 3.1.

10 samples tested in duplicates	Lag Time (minutes)	Peak (nmol.minute)	ttPeak (minutes)	ETP (nmol)
No probe contact	**5.14**	**281.3**	**8.26**	**1750**
SD	**0.41**	**15.54**	**0.53**	**40.44**
Probe contact	**5.09**	**266.8**	**8.01**	**1730**
SD	**0.18**	**11.8**	**0.19**	**33.72**

Table 3.1. pH Meter probe activation measurement
Abbreviations: time to peak (ttPeak), endogenous thrombin potential (ETP).

Although these results were not very conclusive they demonstrated that the glass probe contact alone seemed not to produce any significant changes. This test was not the same as performing pH manipulation. There were also reagents used to change the pH which could trigger sample activation.

3.3. pH regulation and sample activation.

To be able to modify the plasma pH, a physiologic acid (lactic acid) and a base (NaOH) were required; this could affect the stability of plasma components and induce plasma activation.

Both, acid and base were diluted down to 1:10 from the bottle source, this way the pH shock would be reduced. The final concentration used was sodium hydroxide 1.52M and lactic acid 1.16M. Special care had to be taken with PRP plasma samples, due to the fact that platelets are very fragile and very prone to become activated.

3.4. pH regulation strategy.

A study was designed to examine if the sample pH manipulation induced sample activation. Two different approaches were used (Table 3.2.):

- 1st method: the sample pH is changed down to target pH value, measured with the pH meter and then run in the CAT.
- 2nd method: the sample pH is changed and measured in a duplicate sample until the target pH is obtained, then the amount of acid or base used, is recorded and added to the an identical sample but, this time, no sample measurement is taken.

The results were as follows:

Average of 10 samples	Lag Time (minutes)	Peak (nmol.minute)	ttPeak (minutes)	ETP (nmol)
1st method	**4.48**	**398.5**	**6.59**	**2347.9**
standard deviation	0.33	13.8	0.56	257.4
2nd method	**5.17**	**270.5**	**8.46**	**1733.3**
standard deviation	0.13	22.7	0.21	62.9

Table 3.2. Possible sample activation by pH manipulation. 10 samples were used for each method (n=10).
Abbreviations: time to peak (ttPeak), endogenous thrombin potential (ETP).

Comparing results from the 1st and 2nd methods, lag times and time to peak were shorter, peaks were higher and ETPs were increased. Clearly the samples used with the first method were activated by the pH manipulation process. This suggested that the 2nd method was much more appropriate for this study, where samples remained at their baseline values and this approach was used throughout the study.

3.5. Fresh samples compared to frozen & thawed samples.

It is well documented in the literature that the process of sample storage, involving freezing and thawing samples, generates sample activation by cold induced contact activation (Norda *et al*., 2008). This was demonstrated by Palmer *et al*. (1982) who reported shorter times to clot in the PT and APTT in frozen and thawed samples compared to fresh.

Thromboelastometry was used to compare fresh against frozen & thawed samples. Five samples were separated in different aliquots. The fresh ones were run immediately after separation and the rest were frozen (-80°C freezer) and thawed a week later for measurement.

Because PPP was used the EXTEM was the assay of choice. The results between the five fresh samples and the five frozen & thawed F&T) are shown in table 3.3.

5 samples compared	CT (seconds)	CFT (seconds)	α (degrees)	MCF (mm)	AUC (nmol)	MAXV	MAXV-t (seconds)
Fresh	**79**	**2595**	**67.2**	**19.4**	**1972.6**	**11**	**101**
SD	7.55	552	2.59	1.14	86.6	0.71	2.91
Frozen & Thawed	**44.2**	**9526.2**	**69.2**	**13.4**	**1410**	**11.2**	**53.2**
SD	4.49	933.7	1.1	0.89	129.3	0.45	4.71

Table 3.3. Results from fresh samples and frozen & thawed samples Abbreviations: clotting time (CT), clot formation time (CFT), Alpha (α), maximum clot firmness (MCF), area under the curve (AUC), maximum velocity (MAXV), time to maximum velocity (MAXV-t).

These results clearly show the effects of freezing and thawing on clotting profiles.

The clotting time (CT) was almost shortened by a half when using frozen & thawed plasma (from 79sec down to 44.2 sec). Clot formation time (CFT) experienced the opposite effect where times were much more prolonged with the freezed and thawed PPP. MCF, AUC and MAXV-t were higher on the fresh samples. Alpha (α) and MAXV however remained stable.

Overall, these results agree with previously published information about the so called cold-activation phenomenon. Fresh and thawed samples become activated by a temperature effect and also coagulation factor levels (such as FVIII and von Willebrand Factor) can drop down to 50% of the original levels (Favaloro *et al*, 2004). This explains the different changes seen with the ROTEM.

The cold activation phenomenon is responsible for the reduction of the CT. Consequently, the coagulation factors depletion produces a prolonged and lower MCF, AUC and MAXV-t following freezing and thawing of the samples.

To avoid the freezing and thawing effects, this study was performed on fresh samples. The only way to achieve this was by bleeding the healthy volunteers each time before experiments were performed.

4. RESULTS

4.1. Introduction:

The effects of Hypothermia and Acidosis on Haemostasis.

It is well documented that in severe to moderate hypothermia the coagulation screens, such as prothrombin time (PT) and activated partial thromboplastin time (APTT), experience a time prolongation in patients with normal factor levels. (Reed, 1990). This haemostatic delay has been proposed as an increased haemorragic risk (Gubler *et al*, 1994; Breen *et al*., 1988; Armand *et al*., 2003).

In addition, some studies suggested that trauma patients with hypothermia suffer from coagulopathy which increases the mortality rate in the ICU (Patt *et al*, 1988).

In vitro studies investigated the effects of hypothermia (temperatures down to 25°C), showed time prolongation on APTT, PT and thrombin times (TT) (Reed *et al*, 1992). Another study of 45 patients, who were hypothermic and acidotic, revealed that they developed clinically significant bleeding despite adequate blood, plasma, and platelet replacement therapy (Ferrara *et al*, 1990).

4.2. Results

4.2.1. Clotting tests: PT/APTT

To reproduce these findings, the Start4 analyzer was used to perform PT and APTT assays under hypothermic and acidotic conditions respectively and in combination, on 10 different samples from healthy volunteers (Table 4.1.).

Four different experiment conditions were tested:

a) Physiological conditions (Temperature 37°C and pH 7.35)
b) Hypothermia (Temperature 31°C and pH 7.35)
c) Acidosis (Temperature 37°C and pH 6.9)
d) Hypothermia and Acidosis (Temperature 31°C and pH 6.9)

Platelet poor plasma results	PT (seconds)	SD	APTT (seconds)	SD
T37°C / pH 7.35	12.24	0.4	110.53	5.44
T31°C / pH 7.35	17.91	1.84	114.41	11.45
T37°C / pH 6.9	15.17	0.32	147.18	7.17
T31°C / pH 6.9	21.54	1.79	169.27	15.06

Table 4.1. Mean values and standard deviation (SD) on 10 patients for PT and APTT using platelet poor plasma under normal, hypothermic and/or acidotic conditions.
Abbreviations: platelet poor plasma (PPP), prothrombin time (PT), activated partial thromboplastin time (APTT).

Prothrombin times using platelet rich plasma (PRP) were performed without any added phospholipids. This could not be done on the analyzer due to the fact that the PT reagent used for the test (Simplastin HTF) used a lipidated tissue factor. For that reason only APTT was measured on PRP (Table 4.2.).

Platelet rich plasma results	APTT (seconds)	SD
T37°C / pH 7.35	118.2	15.39
T31°C / pH 7.35	136.6	18.11
T37°C / pH 6.9	147.5	20.99
T31°C / pH 6.9	162.4	12.59

Table 4.2. Mean values and standard deviation (SD) on 10 patients for APTT using platelet rich plasma under normal, hypothermic and/or acidotic conditions.
Abbreviations: platelet rich plasma (PRP), prothrombin time (PT), activated partial thromboplastin time (APTT).

4.2.1.1. Discussion

These results confirm those reported by Reed (1990). Hypothermia prolonged the PT by more than 5 seconds and the APTT for almost 4 seconds on the PPP samples. PRP APTT was also prolonged under hypothermic conditions by more than an 18 seconds delay.

Acidotic conditions prolonged the PT for almost 3 seconds and the APTT by more than 36 seconds on the PPP samples. PRP APTT was also delayed by 29 seconds.

The combined effect of hypothermia and acidosis increased further the delays observed above. The PT was prolonged by more than 9 seconds and the APTT by 58 seconds on the PPP samples. The PRP APTT showed a delay of 44 seconds at 31°C of temperature and pH 6.9 compared to normal conditions.

These results showed that either hypothermia or acidosis have an effect on the coagulation process *in vitro*, by delaying PT and APTT clotting times. PT at 31°C/pH7.35 and PT at 37°C/pH6.9 were significant ($p<0.01$) (Table 4.1.). The combined effect of hypothermia and acidosis made the PT/APTT time delays even longer, suggesting a possible cumulative effect of the two parameters over the coagulation process. PT at 31°C/6.9 was very significant ($p<0.01$) (Table 4.1.).

The progressively prolonged PT and APTT times observed *in vitro* under hypothermic and/or acidotic conditions suggest that unless temperature and/or pH are corrected a bleeding tendency may be observed *in vivo*.

4.2.2. Calibrated Automated Thrombogram results

With the clot based assays like PT or APTT, the only parameter that could be measured was the time taken to clot formation.

The CAT not only measured a specific parameter but several in a continuous measurement which ends when there is no more Thrombin to be generated by a sample. This is achieved by a fluorescence technique that allows the measurement to be carried out after the clot has formed. Generally speaking, it gives an overview of the general haemostatic potential of the patient sample.

Several parameters can be measured from this assay:

(i) **Lag Time**: This is the time needed for clot formation. This normally happens when approximately 20nmol of Thrombin are formed.

(ii) **Peak**: It measures the maximum rate of Thrombin formation per unit time.

(iii) **Time to Peak**: It is the time taken to Peak from the reaction start to the Peak formation.

(iv) **Endogenous Thrombin Potential (ETP)**: This measures the total amount of Thrombin that a given sample can generate. It correlates with the area under the curve.

(v) **Start Tail**: starts when there is no more Thrombin left on the sample and the CAT curve reaches the baseline.

(vi) **Velocity**: This parameter measures the thrombotic capacity of the sample. This value is obtained by calculating the slope of the CAT curve by dividing the peak by the difference of time-to-peak minus lag time.

To compare results with the clotting methods, the same conditions were used for assays under hypothermic and acidotic conditions using samples from 10 healthy volunteers:

a) Physiological conditions (Temperature 37°C and pH 7.35)
b) Hypothermia (Temperature 31°C and pH 7.35)
c) Acidosis (Temperature 37°C and pH 6.9)
d) Hypothermia and Acidosis (Temperature 31°C and pH 6.9)

The CAT Results were as follows:

4.2.2.1. Calibrated Automated Thrombogram. Platelet poor plasma

A Lag Time delay was seen with hypothermic and acidotic conditions, respectively, but not statistically significant $p>0.05$. When hypothermia and acidosis were combined the delay was greater (2.55 minutes delay) (Table 4.3) with $p<0.01$ (Table 4.4.).

The overall ETP increased with acidosis and experienced a greater increment with hypothermia and with hypothermia + acidosis (Table 4.3) with $p<0.01$ (Table 4.4.).

Peak changes were not significant. The peak increased with hypothermia alone and showed little change with acidosis or when the two were combined.

The time-to-peak followed the same trend as the lag time for all the different combinations tested.

In hypothermia alone, the velocity slightly decreased but in acidotic conditions it decreased down to 28.7°C. The lowest value for velocity was seen at 31°C and pH6.9 (Table 4.3).

CAT platelet poor plasma results	Lag Time (min)	SD	ETP (nmol)	SD	Peak (nmol.min)	SD	TT Peak (min)	SD	Velocity	SD
37°C /pH 7.35	7.91	1.4	1777	458	191	66.8	13.3	1.79	36.7	15.7
31°C /pH 7.35	8.37	1.4	2681	475	227	60.2	15.1	2.22	35.4	12.9
37°C /pH 6.9	8.22	1.4	1985	432	180	53.2	14.7	1.79	28.7	11.6
31°C /pH 6.9	10.46	1.5	2852	489	202	61.7	19.2	2.21	25.2	13.2

Table 4.3. CAT Results on PPP for normal, hypothermic, acidotic and hypothermic+acidotic conditions.
Abbreviations: platelet poor plasma (PPP), endogenous thrombin potential (ETP), time to peak (TT Peak).
(Statistic results in table 4.4.).

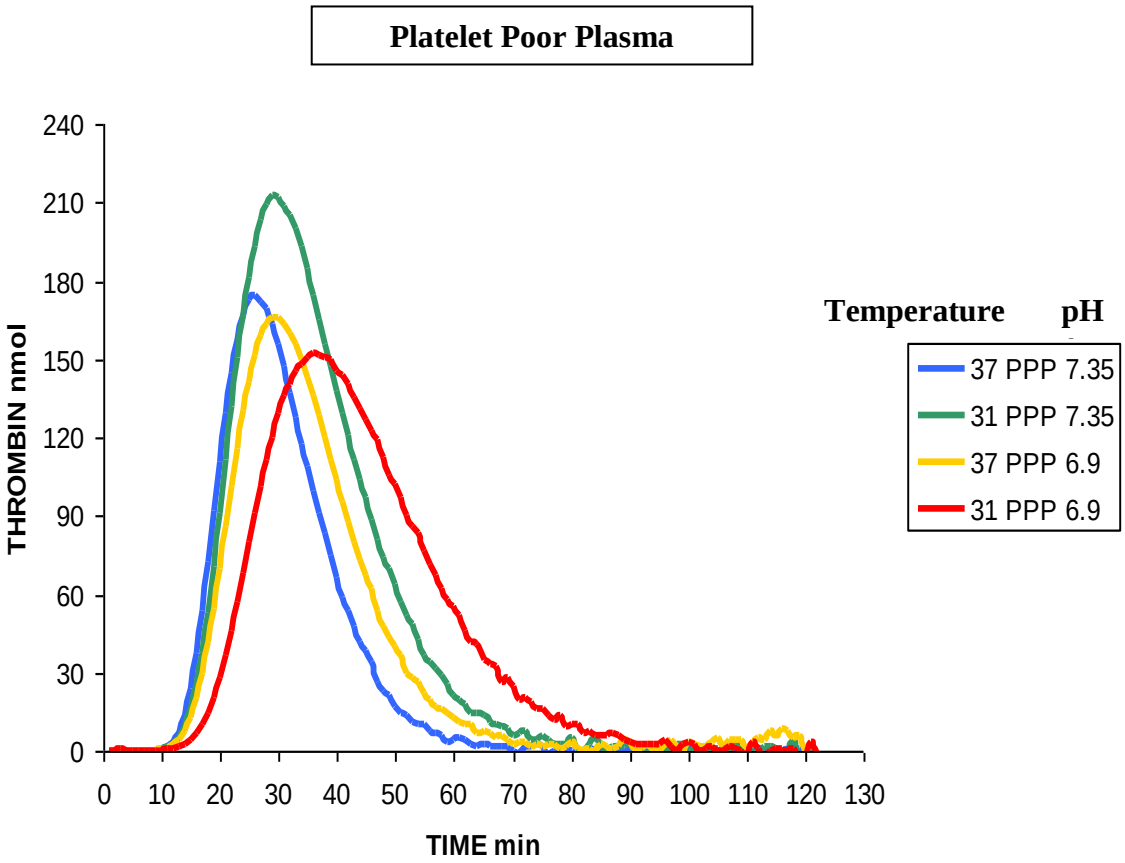


Fig.4.1. PPP Thrombin generation curves for normal, hypothermic, acidotic and hypothermic+acidotic conditions.
Abbreviations: platelet poor plasma (PPP).

The figure (Fig.4.1.) shows four different CAT curves for the thrombin generation assay. Due to the increment in lag time, a shift of the curves to the right was observed when using the abnormal conditions.

In spite of the peak heights, the “area under the curve” or ETP were increased for the three abnormal conditions when comparing it with (blue curve) physiological conditions (T=37°C/pH7.35).

Multiple Comparisons				Tukey HSD		
Dependent Variable	T/pH (I)	T/pH (J)	Mean Difference (I-J)	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Lag Time (min)	37/7.35	31/6.9	-2.55	P<0.01	-4.19	-.91
		31/7.35	-.47	NS	-2.11	1.17
		37/6.9	-.31	NS	-1.96	1.32
ETP (nmol)	37/7.35	31/6.9	-1075.52	P<0.01	-1526.5	-624.48
		31/7.35	-904.49	P<0.01	-1355.5	-453.45
		37/6.9	-207.76	NS	-658.79	243.27
Peak (nmol.min)	37/7.35	31/6.9	-11.04	NS	-74.80	52.73
		31/7.35	-35.96	NS	-99.73	27.80
		37/6.9	11.01	NS	-52.75	74.78
TT Peak (min)	37/7.35	31/6.9	-5.91	P<0.01	-8.35	-3.47
		31/7.35	-1.77	NS	-4.20	.66
		37/6.9	-1.37	NS	-3.81	1.06
Velocity	37/7.35	31/6.9	12.07	NS	-2.59	26.73
		31/7.35	1.31	NS	-13.35	15.97
		37/6.9	7.29	NS	-7.37	21.95
PT (seconds)	37/7.35	31/6.9	-9.30	P<0.01	-10.87	-7.72
		31/7.35	-5.67	P<0.01	-7.24	-4.09
		37/6.9	-2.93	P<0.01	-4.50	-1.35
APTT (seconds)	37/7.35	31/6.9	-41.95	NS	.000	-56.26
		31/7.35	12.91	NS	.090	-1.40
		37/6.9	-19.87	NS	.003	-34.18

Table 4.4. Statistical data on PPP for temperature and pH combinations. This table shows the comparison of data obtained at 37°C/pH7.35 with data obtained at 31°C/pH6.9, 31°C/pH7.35 and 37°C/pH6.9 respectively. This contains data from the different CAT parameters and also PT and APTT times. P values of <0.05 were considered significant (in blue). Non significant p values are expressed as NS (in red). Abbreviations: endogenous thrombin potential (ETP), time to peak (TT Peak), prothrombin time (PT), Activated Partial Thromboplastin Time (APTT). Statistics refer to table 4.3.

4.2.2.2. Calibrated Automated Thrombogram. Platelet rich plasma results

For the lag time, the same trend was seen as with the PPP (Table 4.5.). Time delays were observed using hypothermic and acidotic conditions but when both conditions were combined the delay was even greater ($p<0.01$) (Table 4.6.).

The Endogenous Thrombin Potential (ETP) followed the exact same trend as the PPP, It was increased with both conditions respectively and when run with both, the increment was more pronounced (Table 4.5.). Results were statistically significant for hypothermia ($p<0.01$) and for hypothermia + acidosis ($p<0.01$) (Table 4.6.).

Overall, the peak heights increased under hypothermic and acidotic conditions, but these changes were not significant (Table 4.6.).

The time-to-peak was increased with hypothermia but not with acidosis.

Velocity for PRP did not follow any particular pattern and the results were not significant (Table 4.6.).

CAT platelet rich plasma results	Lag Time (min)	SD	ETP (nmol)	SD	Peak (nmol.min)	SD	TT Peak (min)	SD	Velocity	SD
37°C /pH 7.35	6.28	0.9	1760.55	356	112	46.6	16.1	2.3	11.35	15.4
31°C /pH 7.35	7.52	1.5	2196.1	262	122	36.1	19.3	3.8	10.33	18.1
37°C /pH 6.9	7.12	1.0	2036.75	352	163	42.2	15.4	1.8	19.61	6.2
31°C /pH 6.9	8.58	1.3	2605.75	267	158	21.1	20.1	2.4	13.76	2.8

Table 4.5. CAT Results on PRP for normal, hypothermic, acidotic and hypothermic+acidotic conditions. Abbreviations: platelet rich plasma (PRP), endogenous thrombin potential (ETP), time to peak (TT Peak). (Statistic results in table 4.6.).

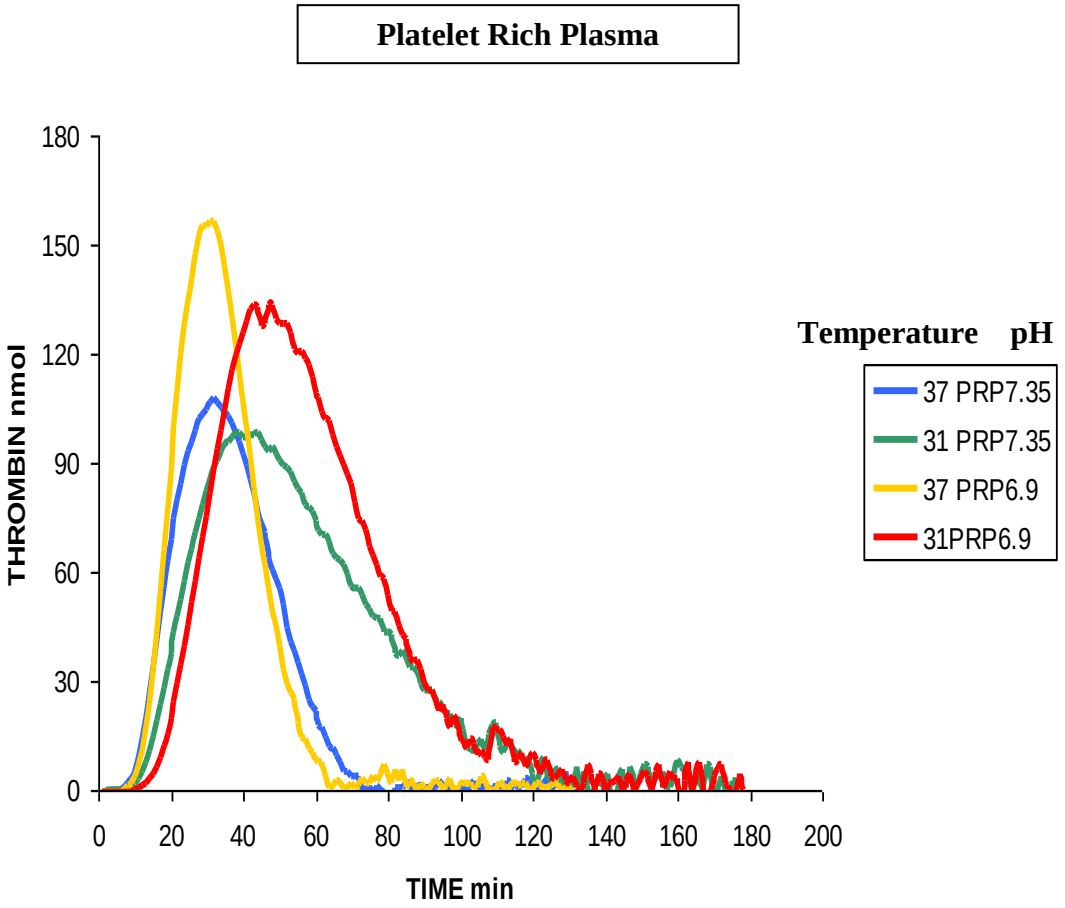


Fig.4.2. PRP Thrombin generation curves for normal, hypothermic, acidotic and hypothermic+acidotic conditions. Abbreviations: platelet rich plasma (PRP).

The results obtained (Fig.4.2.) show that under hypothermic, acidotic and hypothermic + acidotic conditions the CAT curves were moved to the right due to a Lag Time delay.

The results also clearly demonstrated that the “Area under the curve” or ETP is increased in the presence of hypothermic and acidotic conditions, either alone or in combination.

The changes become more obvious when comparing the blue line (T=37°C/pH7.35) and the red line (T=31°C/pH6.9) which shows the changes on the CAT assay when comparing physiological conditions to the combined effect of hypothermia and acidosis.

Multiple Comparisons					Tukey HSD	
Dependent Variable	T/pH (I)	T/pH (J)	Mean Difference (I-J)	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Lag Time (min)	37/7.35	31/6.9	-2.55	P<0.01	-4.19	-.91
		31/7.35	-.47	NS	-2.11	1.17
		37/6.9	-.31	NS	-1.96	1.32
ETP (nmol)	37/7.35	31/6.9	-1075.52	P<0.01	-1526.5	-624.48
		31/7.35	-904.49	P<0.01	-1355.5	-453.45
		37/6.9	-207.76	NS	-658.79	243.27
Peak (nmol.min)	37/7.35	31/6.9	-11.04	NS	-74.80	52.73
		31/7.35	-35.96	NS	-99.73	27.80
		37/6.9	11.01	NS	-52.75	74.78
TT Peak (min)	37/7.35	31/6.9	-5.91	P<0.01	-8.35	-3.47
		31/7.35	-1.77	NS	-4.20	.66
		37/6.9	-1.37	NS	-3.81	1.06
Velocity	37/7.35	31/6.9	12.07	NS	-2.59	26.73
		31/7.35	1.31	NS	-13.35	15.97
		37/6.9	7.29	NS	-7.37	21.95
APTT (seconds)	37/7.35	31/6.9	-41.95	NS	.000	-56.26
		31/7.35	12.91	NS	.090	-1.40
		37/6.9	-19.87	NS	.003	-34.18

Table 4.6. Statistical data on PRP for temperature and pH combinations. This table shows the comparison of data obtained at 37°C/pH7.35 with data obtained at 31°C/pH6.9, 31°C/pH7.35 and 37°C/pH6.9 respectively. This contains data from the different CAT parameters and also APTT times.
P values of <0.05 were considered significant (in blue). Non significant p values are expressed as NS (in red). Abbreviations: endogenous thrombin potential (ETP), time to peak (TT Peak), prothrombin time (PT), Activated Partial Thromboplastin Time (APTT). Statistics refer to table 4.5.
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4.2.2.3. Discussion

The use of two different types of plasma samples, Platelet Poor Plasma (PPP) and Platelet Rich Plasma (PRP) demonstrated that some parameters stayed and behaved equally and others did neither correlate nor followed any particular trend.

The lag time and the ETP were coherent for both types of plasma but the peak, ttPeak and velocity did not show good correlation between PPP and PRP.

With the CAT there seems to be a disparity between some of the results obtained from the different parameters.

When tests were run under a critical condition, the lag times were delayed giving a general and classical coagulopathy profile where the time to clot formation became prolonged.

Conversely, the overall ETP (which is the most important parameter in this assay) did not seem to follow the same bleeding pattern. Much more thrombin was generated under hypothermic or acidotic conditions alone and with the low temperatures and acidic conditions combined; the increment on the ETP became even more conspicuous.

In general, it seems that both parameters (lag time and ETP) are affected by both hypothermic or acidotic conditions on there own but when combining both of them together (hypothermia + acidosis) there was a cumulative effect which exaggerated the changes even more.

These particular observations have not been observed previously in any published literature to date, where a hypocoagulable coagulopathy is described under abnormal conditions, such as hypothermia and acidosis.

These CAT results obtained using hypothermic or acidotic conditions suggest that in spite of having first a delayed lag time, once the thrombin burst occurs it will generate more thrombin which will increase the overall ETP. Therefore a thrombotic profile may be seen instead of a bleeding tendency.

4.2.3. ROTEM® Results

The Assay used the ROTEM® was the EXTEM® for an assessment of clot formation, fibrin polymerisation and fibrinolysis via Tissue factor activation. With the ROTEM® only Platelet Poor Plasma (PPP) was used for this study.

4.2.3.1.ROTEM® results. EXTEM® using Platelet poor plasma

The Clot Formation Times (CFT) demonstrated an average 209.7 seconds delay under hypothermic conditions and 212.8 seconds delay under acidotic conditions. When tested at 31°C/pH 6.9 the CFT was markedly delayed, rising to a mean value of 615.9 seconds, which meant a delay of 437.9 seconds, more than three fold increases compared to physiological conditions (Table 4.7.).

The Alpha (α) parameter only was affected on the two tests when acidotic conditions were tested. Hypothermia did not seem to have any effect.

MCF under physiological conditions was 18.8 mm, when hypothermic and acidotic conditions were used separately. The results showed a slight decrease that did not achieve statistical significance.

When hypothermic and acidotic conditions were combined a greater effect on the MCF (T 31°C/pH 6.9) was observed (15.6 mm) (Table 4.7.).

EXTEM® Platelet poor plasma results	**CFT (seconds)**	**SD**	**ALPHA (degrees)**	**SD**	**MCF (mm)**	**SD**
37°C /pH 7.35	**178.0**	**55.3**	**72**	**3.65**	**18.8**	**3.52**
31°C /pH 7.35	**387.7**	**88.4**	**71.8**	**3.52**	**18.5**	**2.88**
37°C /pH 6.9	**390.8**	**106.9**	**66.4**	**5.23**	**18.1**	**2.42**
31°C /pH 6.9	**615.9**	**153.6**	**62.1**	**9.13**	**15.6**	**2.72**

Table 4.7. ROTEM® results. EXTEM® on PPP for normal, hypothermic, acidotic and hypothermic+acidotic conditions. Abbreviations: clot formation time (CFT), maximum clot firmness (MCF).

4.2.3.1.1. Discussion

All of these results indicate a clear tendency for a coagulopathy in critical conditions. The times to clot were significantly prolonged, the clot formation rates were decreased and clot stability worsened. To summarize, The EXTEM® assay results suggest than under the effects of hypothermia and acidosis there is not only a delay in clot formation but also a much weaker and unstable clot formed, which will be dissolved sooner by the fibrinolytic process.

4.2.3.2. ROTEM® results. (Whole blood)

The ROTEM® was the only technology used in this study that had the capability of performing an assay for whole blood. The assay is called NATEM® and uses whole blood and only calcium was added to trigger the coagulation cascade.

NATEM® Whole blood results	**CT (seconds)**	**CFT (seconds)**	**α (degrees)**	**MCF (mm)**	**AUC (nmol)**	**MAXV**	**MAXV-t (seconds)**
37°C /pH 7.35	**487.3**	**155**	**61.1**	**54.8**	**5494.2**	**9.67**	**600.1**
SD	134	53.5	8.3	5.3	508	2.7	184
31°C /pH 7.35	**584**	**229.7**	**53**	**54.3**	**5469.6**	**6.78**	**686.7**
SD	202	73.9	10.5	6.2	628	2.8	235
37°C /pH 6.9	**549**	**148.2**	**62.4**	**57.4**	**5793.1**	**9.7**	**607.3**
SD	73	28.2	4.4	6.1	648	2.1	61
31°C /pH 6.9	**754.8**	**476.6**	**41**	**43.6**	**4460.4**	**4.7**	**937.5**
SD	243	31.2	15.6	8.7	934	2.4	484

Table 4.8. ROTEM® Results on whole blood using the NATEM® Assay for normal, hypothermic, acidotic and hypothermic+acidotic conditions. Abbreviations: clotting time (CT), clot formation time (CFT), Alpha (α), maximum clot firmness (MCF), area under the curve (AUC), maximum velocity (MAXV), time to maximum velocity (MAXV-t).

CT and CFT were prolonged by lowering temperature and pH independently and very prolonged (CT=265.4 seconds delay/ CFT=321.6 seconds delay) when both hypothermic and acidotic conditions were combined (Table 4.8.).

Hypothermia and acidosis alone did not induce any significant changes on the Alpha angle nor on the MCF, but when combined, Alpha dropped down to 41° and the amplitude of the MCF decreased by 11.2mm.
The AUC calculated by the software only demonstrated a significant decrease to 4460.4 when both acidosis and hypothermia were combined.

4.2.3.2.1. Discussion

The correlation between the results from the whole blood NATEM® assay and the ones performed on PPP with the EXTEM® assay were good.

All of the parameters used in both Assays (CT, CFT, Alpha, and MCF) were completely concordant and confirmed once again that when acidosis and hypothermia are combined haemostasis is affected and a bleeding pattern is observed *in vitro*.
This was also registered when looking at AUC, which was decreased at critical conditions. This close agreement suggests that the effects are plasma based rather than having a cellular influence in this test.

These findings are in agreement with previously published data; a study of Thromboelastometry and acidosis (Engström *et al*. 2006) and a study on hypothermia effects on coagulation using thromboelastometry (Kheirabadi *et al*. 2007), (Dirkmann *et al*. 2008).

5. INTRODUCTION

Having characterized *in vitro* the effects of hypothermia and acidosis on the Haemostatic process, it is necessary to establish the response of normal plasma samples for any given temperature and pH. These reference ranges could then be used to interpret the haemostasis in clinically compromised individuals. This study was performed to derive for any given temperature and pH.

The CAT assay was used to derive these reference ranges. First, to set up reference ranges at different temperatures and secondly, to produce reference ranges at different pH values and ultimately, the reference ranges for combined hypothermic and acidotic conditions.

5.1. REFERENCE CAT RANGES OVER THE TEMPERATURE RANGE 31°C - 41°C

To understand the effects of temperature on the CAT assay, 20 samples from healthy volunteers were assayed at different analytical temperatures. The temperature range was based on physiological temperatures that could be observed during trauma or illness.

Three CAT parameters were examined on this study, namely:
Endogenous thrombin potential (ETP), lag time (LAG) and the peak height of the CAT curve (PEAK).

The temperatures studied ranged from mild hypothermia (31°C, 33°C and 35°C) to moderate/severe hyperthermia (39°C and 41°C).

The aim of this study was to establish the CAT assay ranges for healthy individuals at these different temperatures. The results obtained from this study could be used as standardise results obtained at different body temperatures.

5.1.1. Results using PPP

5.1.1.1. Endogenous Thrombin Potential (ETP)

The table below shows the amount of thrombin produced at any given temperature (Table 5.1.).

ETP at pH 7.35	MEAN (nmol thrombin)	SD
31°C	2680	331
33°C	2188	215
35°C	1939	179
37°C	1792	155
39°C	1425	179
41°C	1138	118

Table 5.1. The effect of temperature on endogenous thrombin potential (ETP).

The ETP increased with hypothermia, reaching a mean value of 2680 nmol of thrombin at 31°C with a p-value <0.01 compared to 37°C (Table 5.4). With hyperthermia, ETP values dropped to 1138 nmol of thrombin produced at 41°C (p-value <0.01) compared to 37°C (Table 5.4).

The results presented in Fig.5.1.demonstrated how the assay temperature affected ETP values using PPP from healthy individuals.

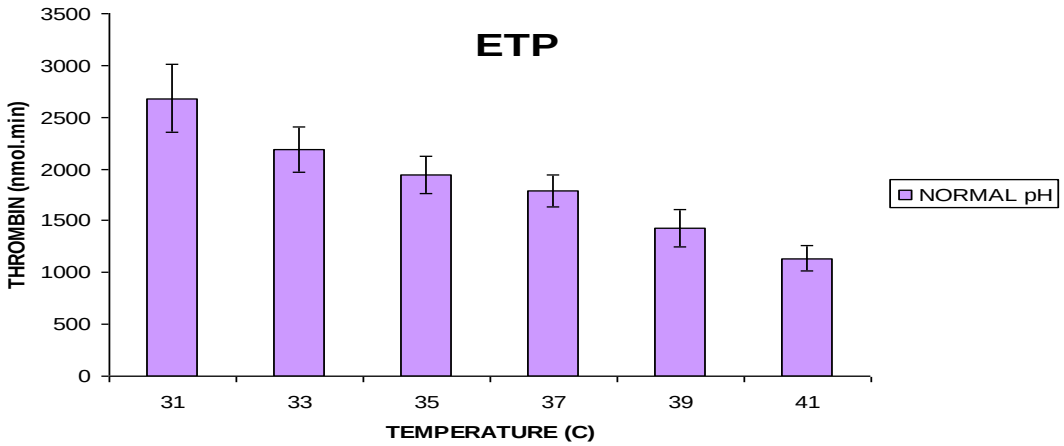

Fig.5.1. The effect of temperature on endogenous thrombin potential (ETP). Results expressed as the mean +/- SD.

5.1.1.2. Lag time (LAG)

The mean lag time observed at normal body temperature was 6.27 min. This lag time increased as the temperature went down (LAG = 8.41 min. at 31°C, p-value <0.01 compared to 37°C (Tables 5.2. and 5.4.)) (Fig.5.2.). On the other hand, the lag time observed under hyperthermic conditions shortened by more than a minute at 41°C (LAG = 5.03 min at 41°C, p-value <0.01 compared to 37°C (Table 5.4.)).

Lag time at pH 7.35	MEAN (minutes)	SD
31°C	8.41	0.89
33°C	7.49	0.84
35°C	6.73	1.2
37°C	6.27	1.42
39°C	6.06	1.59
41°C	5.03	0.67

Table 5.2. The effect of temperature on Lag times.

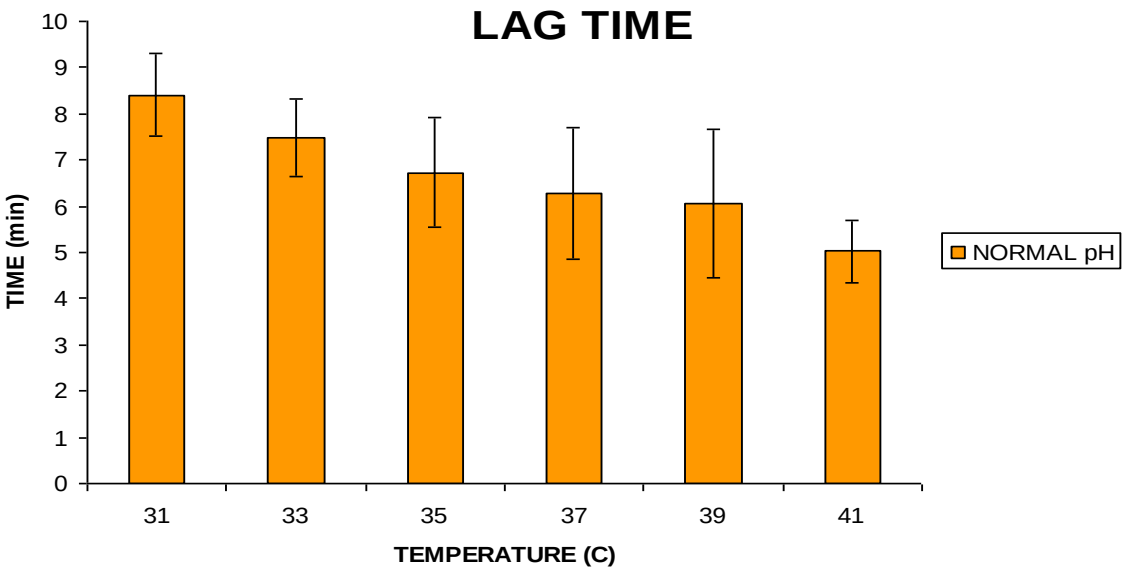

Fig. 5.2. The effect of temperature on Lag times.
Results expressed as the mean +/- SD.

5.1.1.3. Peak height (PEAK)

The peak height results obtained followed a similar trend to the ETP values (Table 5.3.) (Fig. 5.3.). PEAK values in hypothermic conditions were much higher than those noted under hyperthermic conditions. However, the only significant peak values were observed at 31°C ($p<0.05$) and at 41°C ($p<0.01$) (Table 5.4.).

PEAK at pH 7.35	MEAN (nmol.minute)	SD
31°C	216	54.8
33°C	201.3	40.7
35°C	200	38.5
37°C	173.7	31.4
39°C	148.5	50.4
41°C	126.1	35.1

Table 5.3. The effect of temperature on Peak.

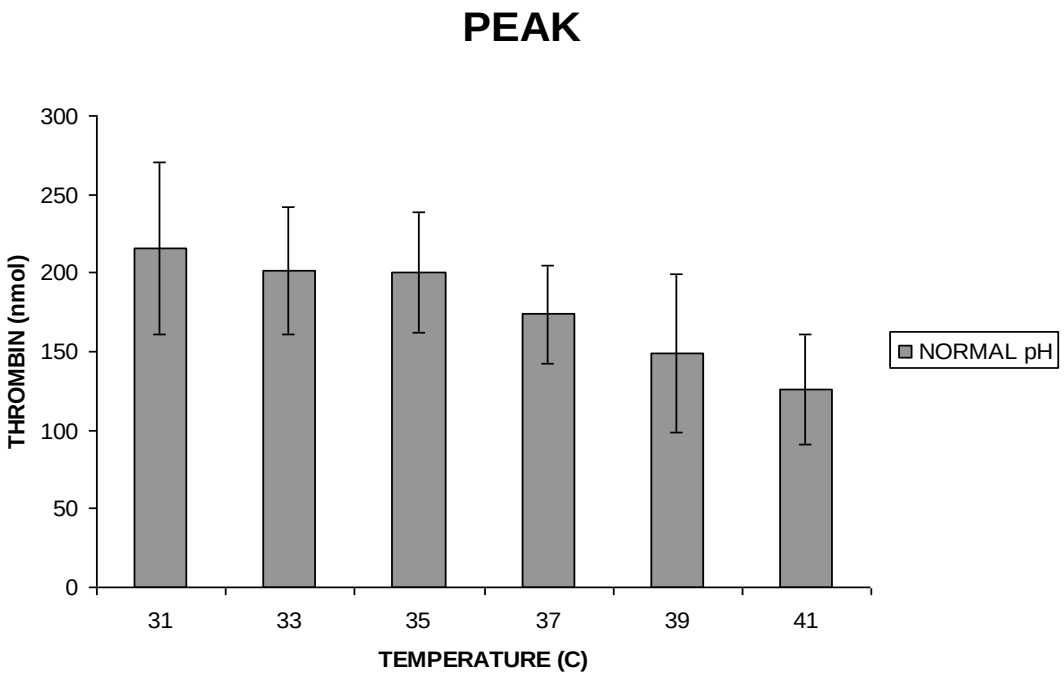

Fig. 5.3. The effect of temperature on Peak. Results expressed as the mean +/- SD.

Multiple Comparisons - Tukey HSD -					95% Confidence Interval	
Dependent Variable	Temperature (I)	Temperature (J)	Mean Difference (I-J)	**p-value**	Lower Bound	Upper Bound
Lag Time (min)	**37**	**31**	-2.14	**P<0.01**	-3.199	-1.07
		33	-1.22	**P<0.01**	-2.280	-.15
		35	-0.46	**NS**	-1.522	.60
		39	0.21	**NS**	-.849	1.27
		41	1.24	**P<0.01**	.161	2.31
ETP (nmol)	**37**	**31**	-887.73	**P<0.01**	-1078.09	-697.36
		33	-395.67	**P<0.01**	-586.03	-205.30
		35	-146.53	**NS**	-336.89	43.83
		39	367.68	**P<0.01**	177.32	558.04
		41	653.94	**P<0.01**	463.58	844.3
Peak (nmol.min)	**37**	**31**	-42.35	**P<0.05**	-81.49	-3.2
		33	-27.62	**NS**	-66.76	11.52
		35	-26.32	**NS**	-65.46	12.82
		39	25.18	**NS**	-13.96	64.32
		41	47.59	**P<0.01**	7.93	87.24

Table 5.4. Statistical data on reference CAT ranges for the temperature studies.This table shows the comparison of data obtained at 37°C with data obtained at 31°C,33°C, 35°C, 39°C and 41°C respectively. P values of <0.05 were considered significant (in blue). Non significant p values are expressed as NS (in red). Abbreviations: endogenous thrombin potential (ETP).

5.2. REFERENCE CAT RANGES OVER THE pH range 6.9-7.5

This study also examined the effects that different pH values exerted on the CAT Assay. Samples from healthy volunteers were assayed. The pH range is based on physiological values that the human body can sometimes experience in trauma or illness (Lier *et al*., 2008; Demirjian *et al*., 2008).

Three CAT parameters were looked at on this study, namely:
Endogenous thrombin potential (ETP), lag time (LAG) and the peak height of the CAT curve (PEAK).

Four different pH values were used: pH 6.9, pH 7.1, pH 7.35 and pH 7.5.

The aim of this study was to establish the CAT assay ranges for healthy individuals at these different pH values. The results obtained from this study could be used to standardise results from patients.

5.2.1. Results on PPP

5.2.1.1. Lag Time

Acidosis increased the lag time by almost 2 minutes (8.225 min. at pH 6.9) ($p<0.05$) (Table 5.8.), compared with the lag time at physiological pH (6.227 min. at pH 7.35). At pH 7.5 lag time shortened to 5.29 minutes (Table5.5.) (Fig. 5.4.).

LAG TIME	MEAN (minutes)	SD
pH 6.9	8.225	1.31
pH 7.1	7.087	0.7
pH 7.35	6.277	1.93
pH 7.5	5.29	1.27

Table 5.5. The effects of pH on lag time results. Results are expressed as mean ±SD of 10 values.

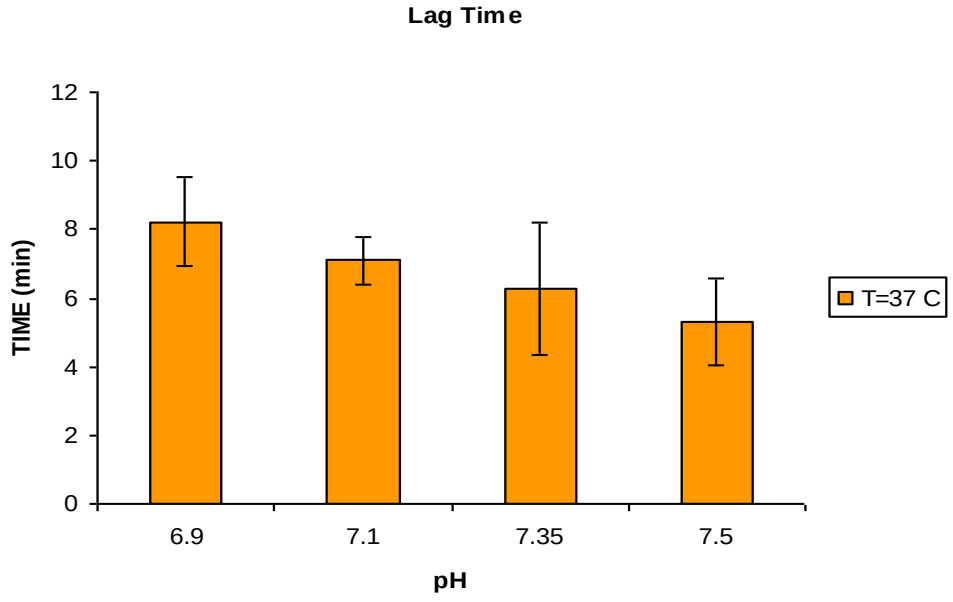

Fig. 5.4. The effect of pH on lag time variations. Results expressed as mean ±SD of 10 estimations.

5.2.1.2. Endogenous Thrombin Potential (ETP)

The table below (Table 5.6) shows the amount of thrombin produced at different pH values.

ETP	MEAN (nmol thrombin)	SD
pH 6.9	1984.8	684.3
pH 7.1	1935.2	592.7
pH 7.35	1789.8	570.1
pH 7.5	1429.5	496.2

Table 5.6. Endogenous thrombin potential (ETP) results for different pH values.

At a pH of 6.9 ETP increased to a maximum value of 1984.8nmol. Conversely, when the pH was alkaline, the ETP decreased down to 1429 nmol (pH 7.5) ($p<0.05$, compared to pH 7.35) (Table 5.8.).

The results (Fig. 5.5.) show how the assay pH affected the ETP using PPP from healthy volunteers.

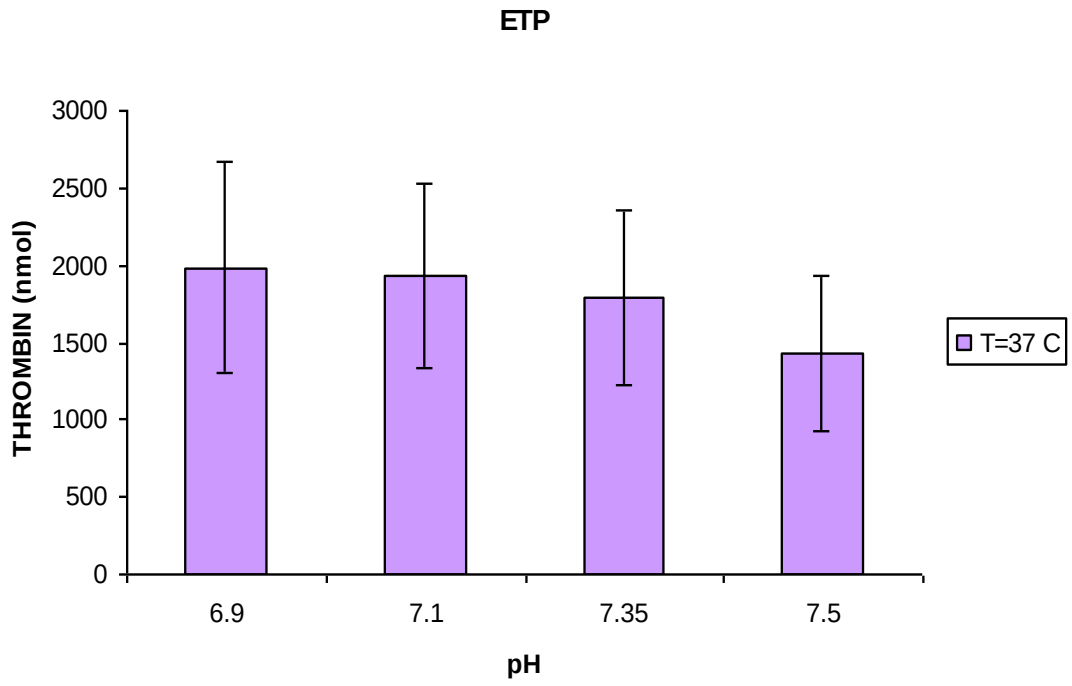

Fig. 5.5. The effect of pH on ETP. Results expressed as mean ±SD. Abbreviations: endogenous thrombin potential (ETP).

5.2.1.3. Peak height (PEAK)

As the following Table shows (Table 5.7.), peak height of the CAT curve was not affected by the assay pH. The PEAK remained almost identical at the four pH values tested.

PEAK	MEAN (nmol.minute)	SD
6.9	179.8	68.6
7.1	174.3	80.2
7.35	173.7	61.1
7.5	177.9	76.7

Table 5.7. PEAK results for different pH values.

Multiple Comparisons - Tukey HSD -					95% Confidence Interval	
Dependent Variable	Temperature (I)	Temperature (J)	Mean Difference (I-J)	**p-value**	Lower Bound	Upper Bound
Lag Time	**7.35**	**6.9**	-1.95	**P<0.05**	-3.61	-0.29
		7.1	-0.81	**NS**	-2.47	0.85
		7.5	0.99	**NS**	-0.67	2.65
ETP	**7.35**	**6.9**	-194.9	**NS**	-495.67	105.85
		7.1	-145.35	**NS**	-446.11	155.41
		7.5	360.36	**P<0.05**	59.59	661.12
Peak	**7.35**	**6.9**	-6.13	**NS**	-70.64	58.38
		7.1	-0.59	**NS**	-65.10	63.92
		7.5	-4.19	**NS**	-68.70	60.32

Table 5.8. Statistical data on reference CAT ranges for the pH studies. This table shows the comparison of data obtained at pH 7.35 with pH 6.9, pH 7.1 and pH 7.5 respectively. P values of <0.05 were considered significant (in blue). Non significant p values are expressed as NS (in red). Abbreviations: endogenous thrombin potential (ETP).

5.3. REFERENCE RANGES AT pH 7.1

The reference ranges presented in this chapter could be used as the reference to be compared when measuring any given sample with the CAT Assay.

In the next chapter, samples obtained from Addenbrooke's Hospital Intensive Care Unit patients were used and tested.

To be able to compare the use these normal ranges in the ICU set of samples, a combination of assay temperature and assay pH were required. Based on the average conditions of the 10 ICU samples tested, it was clear that ranges based upon a fixed pH of 7.1 combined with varying degrees of acidosis was required (Table 5.9.).
The table below shows the reference ranges set for hypothermia (temperature from 31°C to 37°C) and pH 7.1(Table 5.9.).

Lag time at pH 7.1	**MEAN** (min)	**SD**
31°C	8.846	0.62
33°C	8.063	0.66
35°C	7.854	0.69
37°C	7.087	0.73
ETP at pH 7.1	**MEAN** (nmol)	**SD**
31°C	2543	227.7
33°C	2206	251.2
35°C	2130	202
37°C	1935	121.8
PEAK at pH 7.1	**MEAN** (nmol.min)	**SD**
31°C	190.2	39.3
33°C	182.9	48.1
35°C	175.2	53.5
37°C	174.3	65.7

Table 5.9. Lag time, ETP and peak results for hypothermia and pH 7.1. Abbreviations: endogenous thrombin potential (ETP).

As noted previously, parameters such as lag time (Fig. 5.6.) and ETP (Fig. 5.7.) were much higher during hypothermic conditions at pH 7.1. The peak heights did not experience significant changes with the drop in temperature at pH 7.1. (Fig. 5.8.).

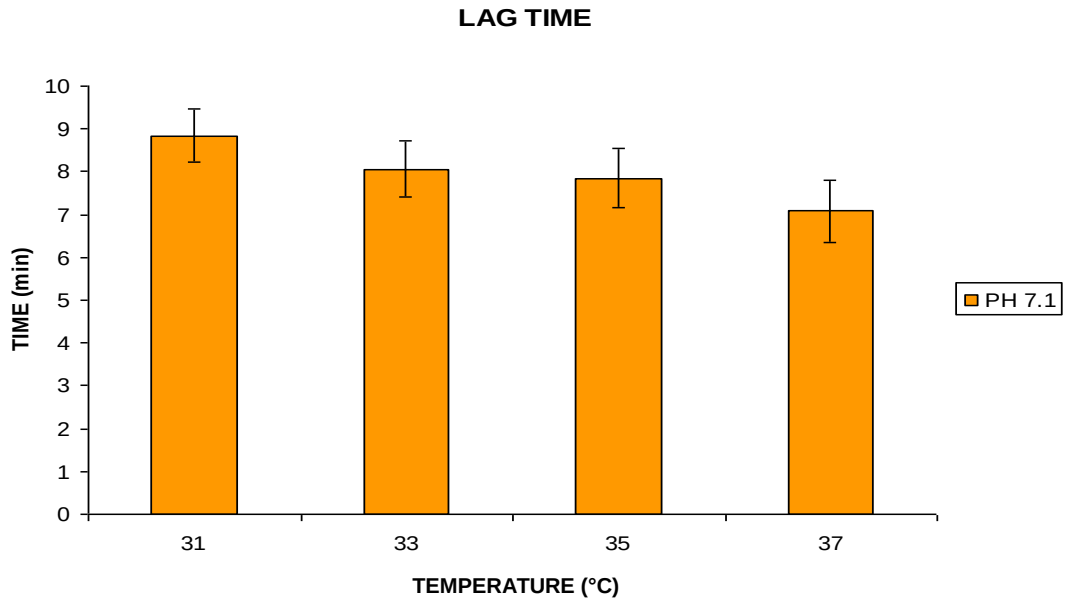

Fig. 5.6. Lag time variations with temperature at pH 7.1. Results expressed as mean ±SD.

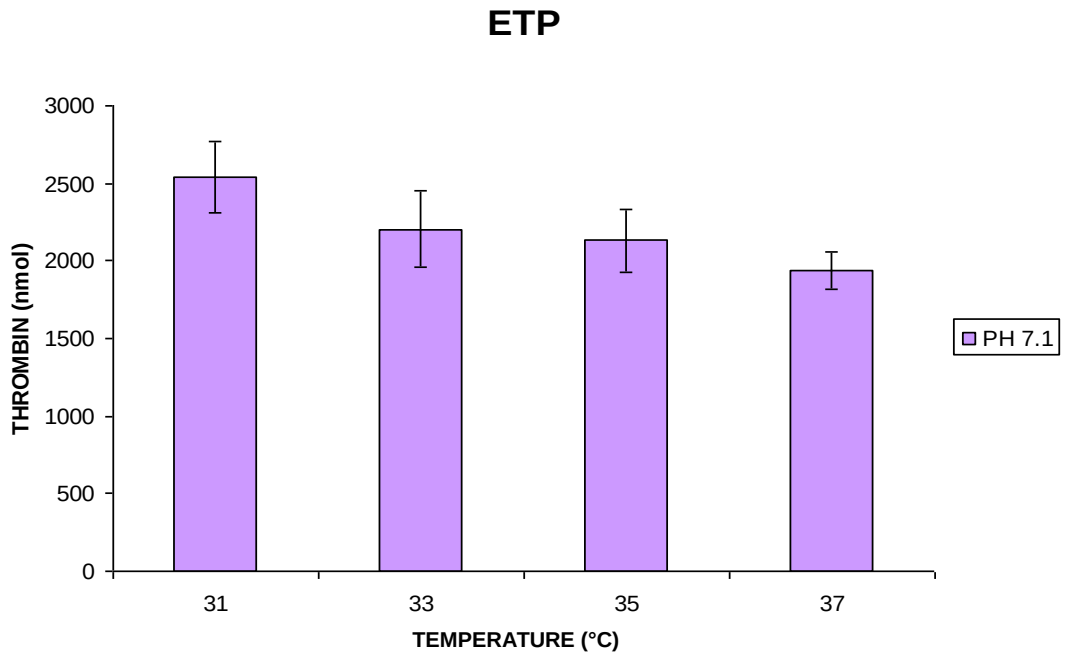

Fig. 5.7. ETP variations with temperature at pH 7.1. Results expressed as mean ±SD. Abbreviations: endogenous thrombin potential (ETP).

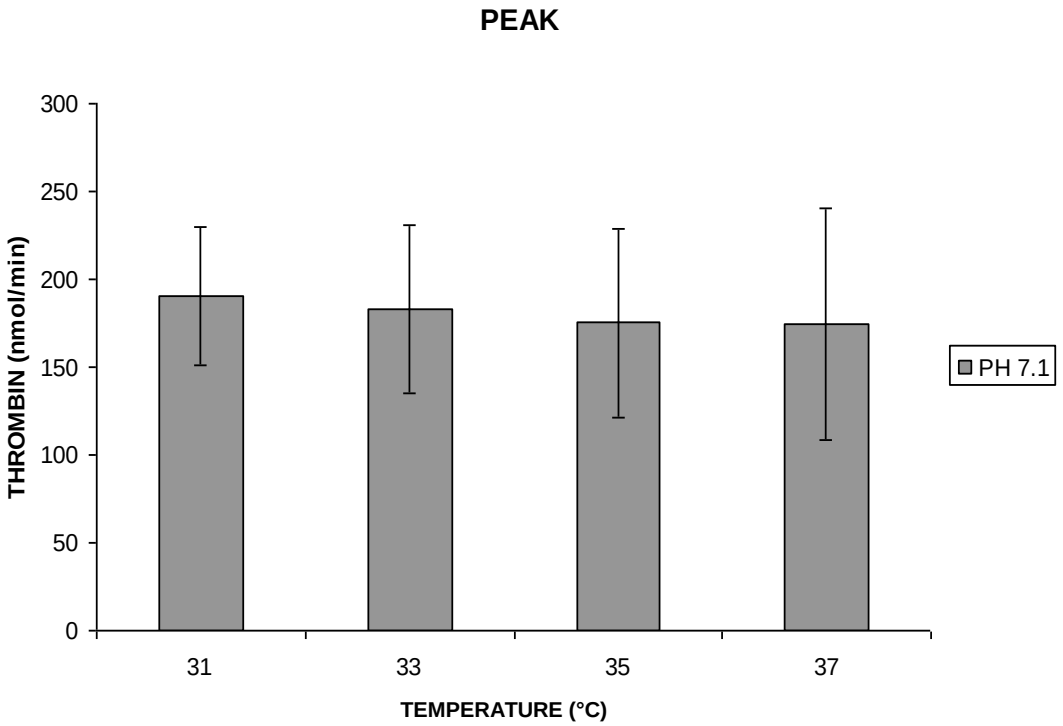

Fig. 5.8. Peak variations with temperature at pH 7.1. Results expressed as mean ±SD.

6. STUDIES ON ICU SAMPLES

This chapter looks at data obtained from 17 patients who were admitted to the Intensive Care Unit at Addenbrooke's Hospital. Consent was obtained for sample testing in the laboratory with all the patient details being anonymized.

Samples were collected in S-monovette® 9NC (PFA). These tubes were chosen because they contain a buffer which will preserve the original pH of the sample. Corn Trypsin Inhibitor (12.66 µl CTI) was also added to each tube before use. This gave a final concentration of 1.45µmol/L of CTI.

The selection process used for the 17 patients in the ICU was based on two parameters used on the APACHE Scoring System: Temperature and pH.

The requirements were simple, patients had to experience some degree of hypothermia and/or acidosis when admitted to the ICU and when the samples were taken.

The patients who fitted these criteria were selected and the temperature and pH of the patient was noted at the time of bleeding.

Out of the 17 patients received from ICU, only 10 were finally used due to heparin contamination. The heparin contaminated samples gave a completely flat line on the CAT when analyzed; therefore no data was obtained from these samples.

A full description of the temperatures and pH of the 10 patient samples used from ICU is given in the table below:

ICU SAMPLES	Temperature	pH
1	36.1	7.12
2	35.4	7.21
3	35.7	7.15
4	36.1	7.04
5	35.9	7.22
6	34.5	7.06
7	34.8	7.1
8	36	7.15
9	35.8	7.14
10	35.6	7.13
Mean	**35.59**	**7.132**
SD	**0.546606**	**0.057116**

Table 6.1 Temperature and pH of ICU samples.
Abbreviations: intensive care unit (ICU).

6.1. RESULTS

Samples were tested as soon as they arrived from ICU using the CAT Assay. PPP and PRP were prepared from whole blood.

To study the effects of hypothermia and acidosis on the patient's clotting profile, two parallel assays were designed:

a) CAT assay of patient's PPP and PRP at its original temperature and pH.

b) CAT assay of patient's PPP and PRP at a corrected analytical temperature (37°C) and physiological pH (7.35).

6.1.1. PRP – CAT

Table 6.2 shows the CAT parameters for the two different assays described above (Table 6.1). The results are presented as the average values of the patient results. pH was always measured at a room temperature. For correction or change of sample pH, sodium hydroxide (1.52M) or lactic acid (1.16M) was used. Assay temperatures were set on the analyzer.

ICU PRP (Original T/pH)	Lag Time (min)	ETP (nmol)	PEAK (nmol.min)	TT PEAK (min)	Start tail (min)
Mean	10.65	1910	107	19.48	65.1
SD	1.5	156.9	10.1	1.47	6.3
ICU PRP (37°C /pH 7.35)	**Lag Time (min)**	**ETP (nmol)**	**PEAK (nmol.min)**	**TT PEAK (min)**	**Start tail (min)**
Mean	5.44	1684.1	90.3	14.36	37.9
SD	0.99	97.7	4.71	1.52	3.38

Table 6.2. CAT results for ICU with PRP. Abbreviations: intensive care unit (ICU), platelet rich plasma (PRP), endogenous thrombin potential (ETP), time-to-peak (TT PEAK).

When comparing the average values of the ICU patients in their original hypothermic acidotic state, with the values measured, when body temperature and pH were restored back to standardised conditions (T=37°C, pH=7.35) a number of key changes were observed:

i. A marked decrease in the lag time from 10.65 min to 5.44 min was seen when temperature and pH were standardised.
ii. The ETP decreased from 1910 to 1684 nmol of thrombin.
iii. The peak was slightly lower.
iv. The time-to-peak was also shortened.

Independent Samples Test (PRP)	t-test for Equality of Means				
	T	p-value	Mean Difference	95% Confidence Interval of the Difference	
				Lower	Upper
Lag time (min)	9.136	**P<0.01**	5.21	4.01	6.41
ETP (nmol)	3.868	**P<0.01**	226.1	103.3	348.89
PEAK (nmol.min)	4.756	**P<0.01**	16.69	9.09	24.28
TT PEAK (min)	7.641	**P<0.01**	5.12	3.71	6.52
Start tail (min)	12.00	**P<0.01**	27.18	22.42	31.94

Table 6.3: Independent Samples t-test on the data in table 6.2. P values of <0.05 were considered significant (in blue). Abbreviations: platelet rich plasma (PRP), endogenous thrombin potential (ETP).

To visualize how this temperature and pH correction affected the CAT, a CAT profile (obtained from the average values of the 10 samples) was derived for both the original temperature and pH data, and for the corrected parameters. (Fig.6.1)

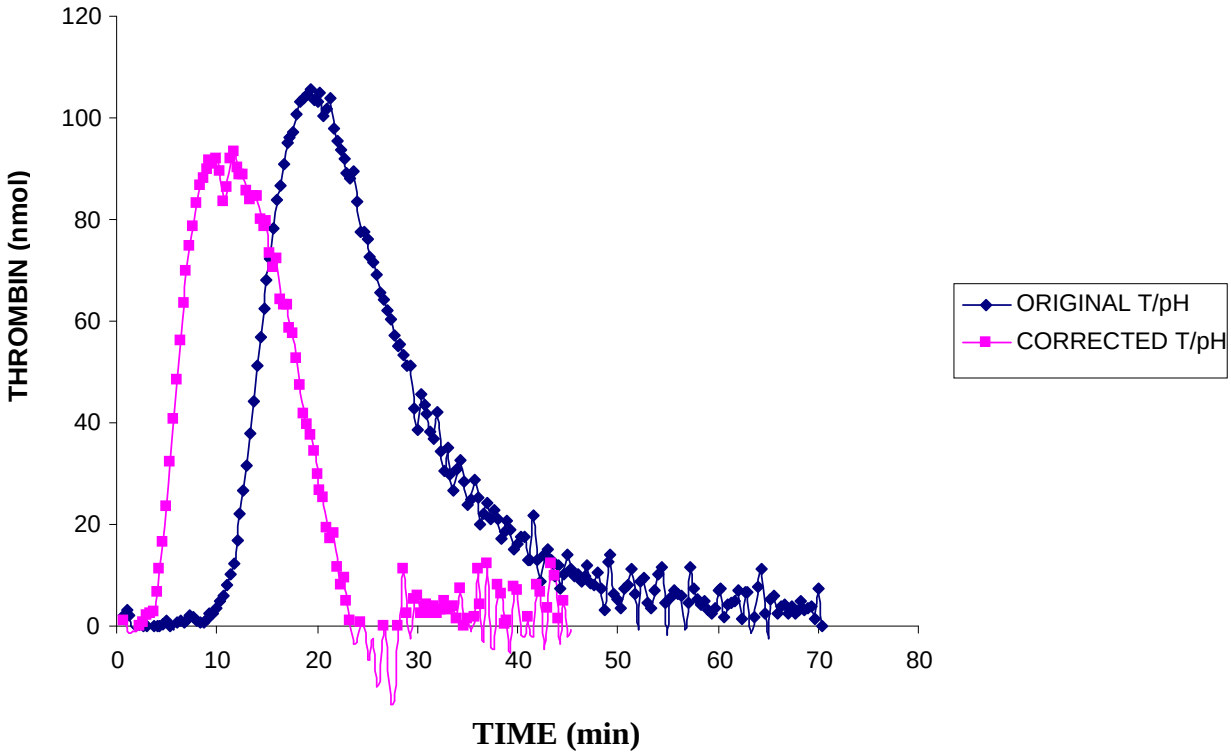

Fig. 6.1. PRP CAT curves for the original and corrected temperature and pH data. Abbreviations: platelet rich plasma (PRP), calibrated automated thrombogram (CAT).

6.1.2. PPP – CAT

ICU PPP (Original T/pH)	Lag Time (min)	ETP (nmol)	PEAK (nmol.min)	TT PEAK (min)	Start tail (min)
Mean	10.77	1629.7	144.6	15.5	50.2
SD	0.78	181.4	20.9	2.6	4.24
ICU PPP (37°C /pH 7.35)	**Lag Time (min)**	**ETP (nmol)**	**PEAK (nmol.min)**	**TT PEAK (min)**	**Start tail (min)**
Mean	4.84	1425.4	111.3	8.34	33
SD	0.63	136.8	16.1	0.61	6.1

Table 6.4. CAT results for ICU using PPP. Abbreviations: intensive care unit (ICU), platelet poor plasma (PPP), endogenous thrombin potential (ETP), time-to-peak (TT PEAK).

Table 6.4 shows the different CAT parameters for assays performed under standardised conditions and the original hypothermic and acidotic conditions.

When comparing the average values of the ICU patients in their original hypothermic acidotic state with the values measured (as done before with the PRP parameters), various important differences were again observed. These are summarized below:

i. The Lag Time dropped dramatically from 10.77 min down to 4.84 min.
ii. The ETP decreased slightly from 1629.7 to 1425.4 nmol of Thrombin.
iii. The Peak was 30nmol/min lower than under standardised conditions.
iv. The TT Peak was reduced from 15.5 to 8.34 min with the correction.

Independent Samples Test (PPP)	t-test for Equality of Means				
	t	p-value	Mean difference	95% Confidence Interval of the Difference	
				Lower	Upper
Lag time (min)	18.65	**P<0.01**	5.93	5.26	6.6
ETP (nmol)	2.84	**P<0.01**	204.29	53.34	355.23
PEAK (nmol.min)	3.98	**P<0.01**	33.28	15.73	50.82
TT PEAK (min)	8.52	**P<0.01**	7.21	5.32	9.09
Start tail (min)	7.30	**P<0.01**	17.2	12.25	22.15

Table 6.5: Independent Samples t-test on the data in table 6.4. P values of <0.05 were considered significant (in blue). Abbreviations: platelet poor plasma (PPP), endogenous thrombin potential (ETP).

The figure below demonstrates clearly how the temperature/pH correction affected the measurements (Fig.6.2)

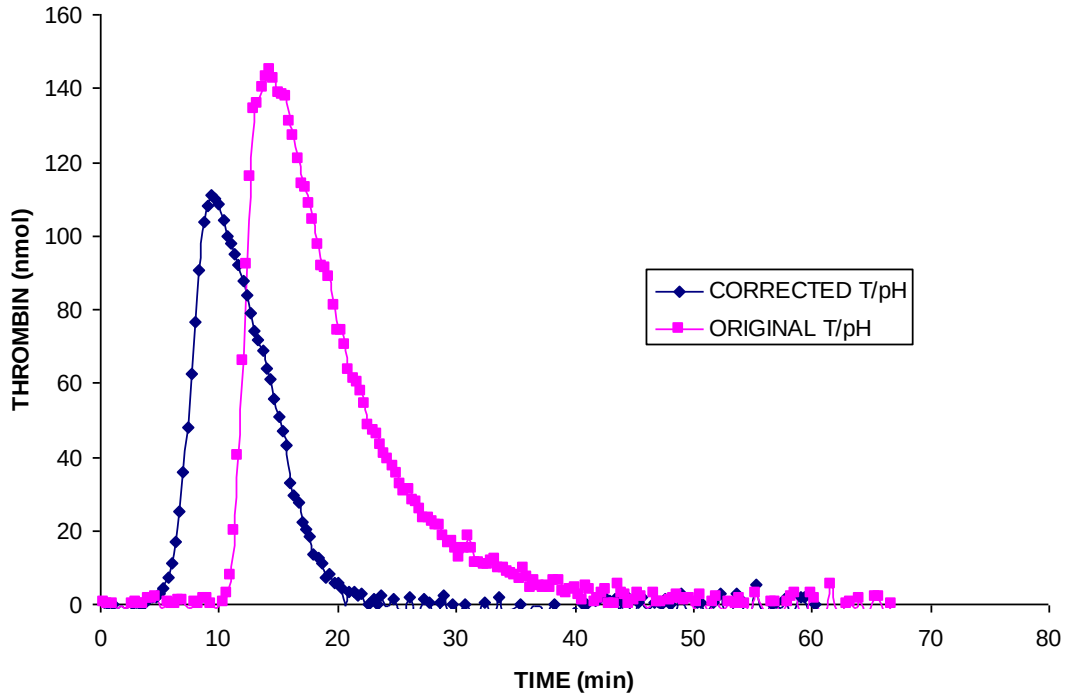

Fig. 6.2. PPP CAT curves for the original and corrected temperature and pH data. Abbreviations: platelet poor plasma (PPP), calibrated automated thrombogram (CAT).

6.2. ICU CAT results compared to CAT Reference ranges

The three most important parameters of the CAT Assay (Lag time, ETP and peak) were chosen for the comparison of the ICU results with the reference ranges obtained in the previous chapter.

Two different comparisons were performed:

a) ICU results against reference ranges at 35°C and a pH of 7.1.
b) ICU results against reference ranges at 37°C and a pH of 7.35.

The graphs below show the a) and b) comparisons for lag time (Fig. 6.3), for ETP (Fig. 6.4) and the peak height (Fig. 6.5).

6.2.1. Lag Time. ICU against Reference ranges

The lag times of the ICU samples for temperature 35°C and pH 7.1 were much longer than those obtained in healthy volunteers.

When measured under standardised conditions (37°C/pH 7.35), the ICU sample lag times were much closer, if not shorter in some cases, to the reference ranges.

These results obtained at a 35°C and pH 7.1 indicated that ICU samples are hypo-coagulable, but when conditions were standardised, the pattern appeared to suggest a hyper-coagulable state.

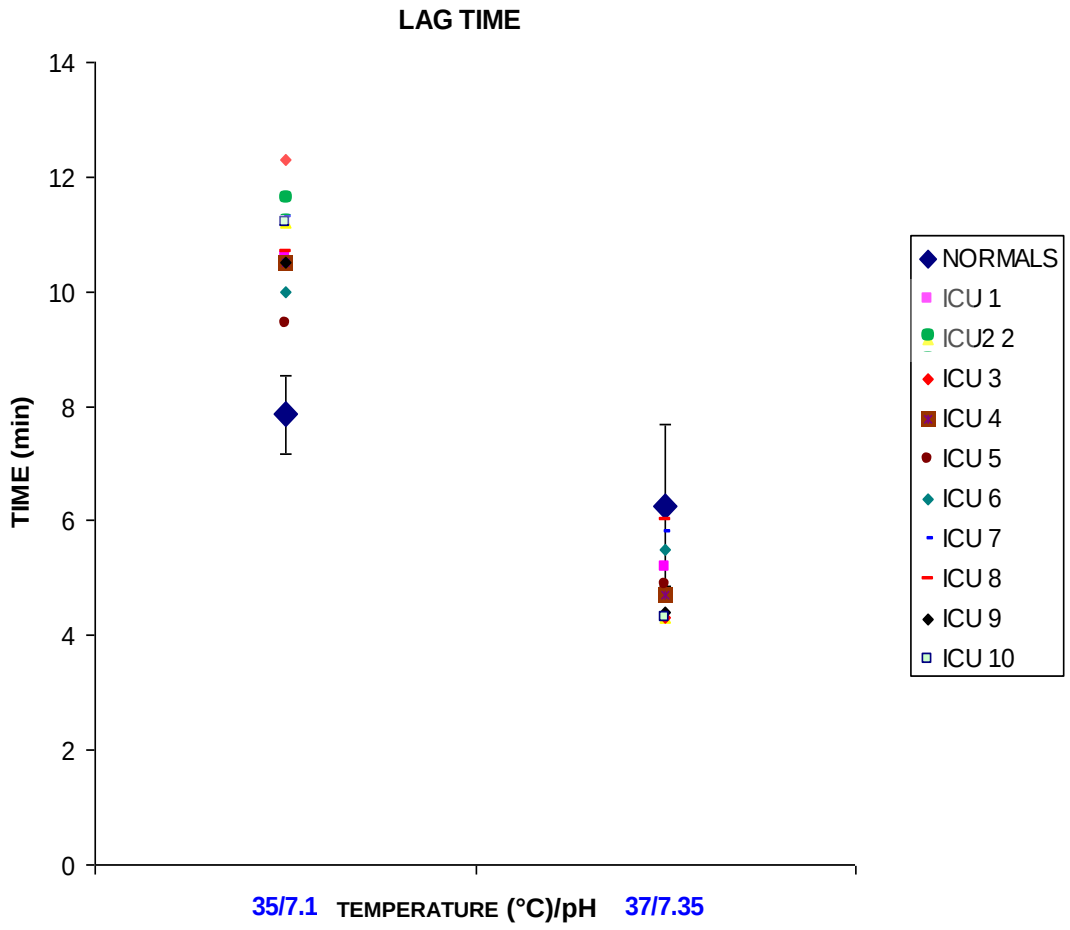

Fig.6.3. ICU results against reference ranges for lag time. Reference values are expressed as the mean +/- SD.

6.2.2. ETP. ICU against Reference ranges

The same pattern of results was seen at 35°C /pH 7.1 as seen at 37°C /pH 7.35. ICU ETP seems to be closer to the reference ranges at 37°C /pH 7.35 than at 35°C /pH 7.1, where values seem to be more dispersed. In both cases, the trend of the ICU is to have a lower ETP than those in healthy volunteers.

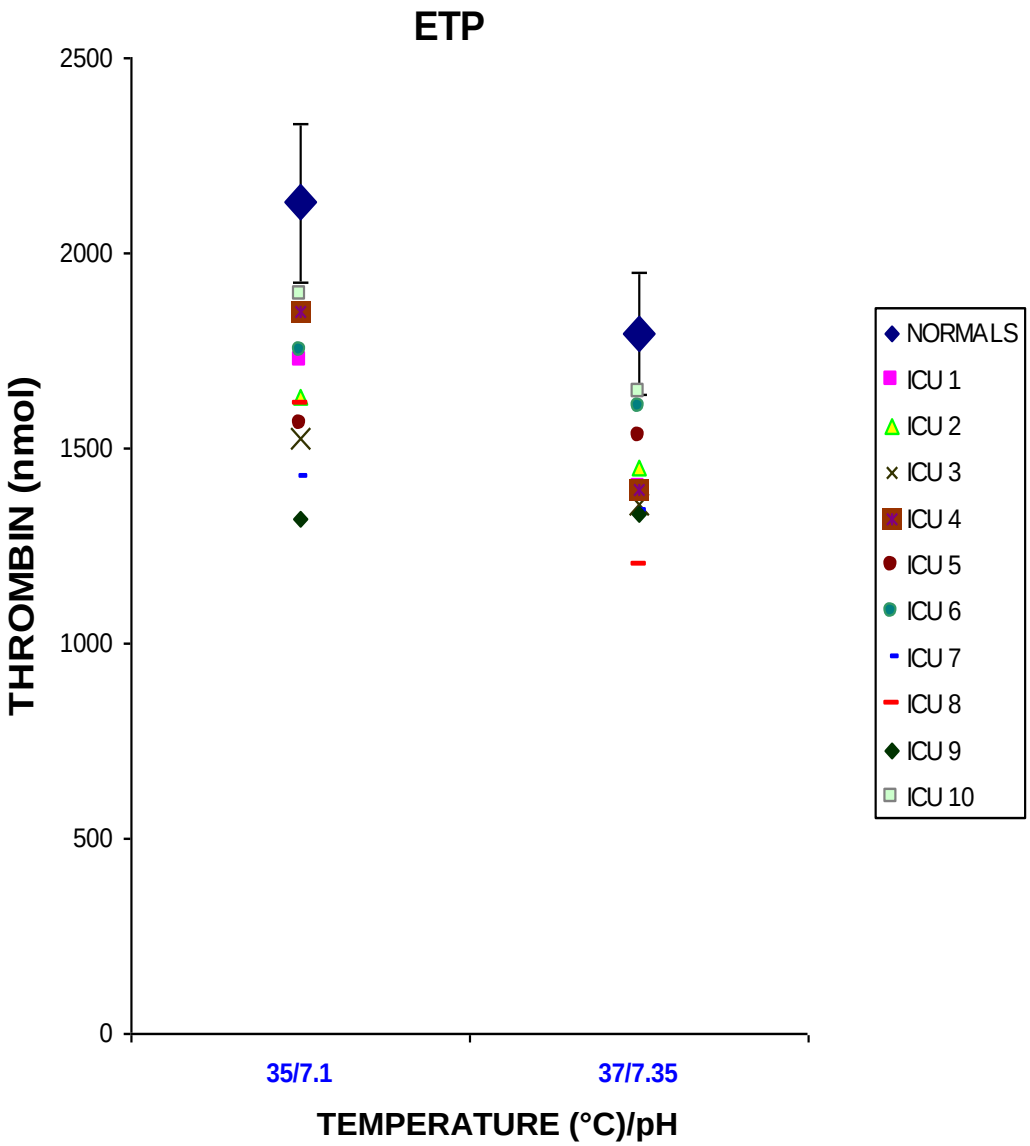

Fig.6.4. Reference ranges against ICU results for ETP values. Reference values are expressed as the mean +/- SD. Abbreviations: endogenous thrombin potential (ETP).

6.2.3. Peak. ICU against Reference ranges

The peak height of the ICU samples at 35°C/pH 7.1 was similar to the reference range values under the same conditions. When tested at 37°C/pH 7.35 ICU peak values were below reference range. This indicates that ICU samples have a hypo-coagulable tendency when measured at standardised pH and temperature but seem to be normal when tested at their original conditions.

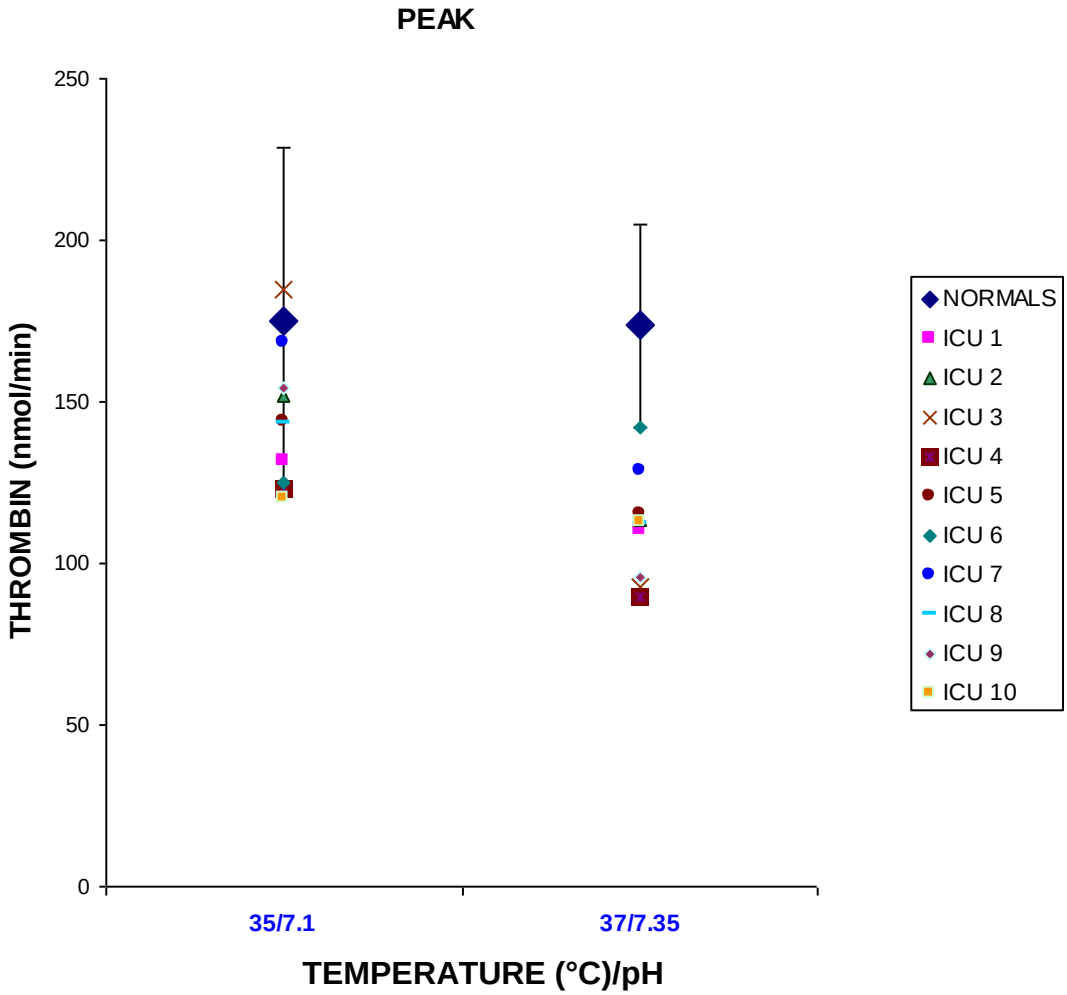

Fig.6.5. Reference ranges against ICU results for peak values. Reference values are expressed as the mean +/- SD.

Independent Samples Test 35°C/pH 7.1		t-test for Equality of Means				
		t	p-value	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Lag time (min)	Equal variances assumed	8.80	P<0.01	2.92	2.22	3.61
ETP (nmol)	Equal variances assumed	-5.82	P<0.01	-500.50	-680.90	-320.09
PEAK (nmol.min)	Equal variances not assumed	-1.68	NS	-30.62	-70.30	9.05

Table 6.6. t-test for comparison of means between ICU samples and healthy volunteers samples at 35°C and pH 7.1.
Abbreviations: endogenous thrombin potential (ETP).

Independent Samples Test 37°C/pH 7.35		t-test for Equality of Means				
		t	p-value	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Lag time (min)	Equal variances assumed	-2.00	NS	-1.34	-2.74	.07
ETP (nmol)	Equal variances assumed	-4.75	P<0.01	-364.44	-525.63	-202.25
PEAK (nmol.min)	Equal variances assumed	-4.91	P<0.01	-62.40	-89.08	-35.71

Table 6.7. t-test for comparison of means between ICU samples and healthy volunteers samples at 37°C and pH 7.35.
Abbreviations: endogenous thrombin potential (ETP).

6.3. HEPARIN SAMPLE REMOVAL

Due to problems with heparin contamination, some of the samples received from the ICU could not be measured on the CAT and therefore, the data was lost and more importantly a valuable sample was wasted.

Heparin is an anticoagulant with several effects on the coagulation cascade. The most important one is the increase in the thrombin inhibitory potency of ATIII by 1000 fold (Blauhut *et al*., 1980). ATIII inhibits thrombin + FIXa, FXIa & FXIIa.
Heparin contamination prolongs the APTT, to a lesser extent the PT and also affects thrombin generation on the CAT Assay, which is much more sensitive than traditional coagulation tests on detecting heparin in the sample (Gatt, A. et al., 2008).

A possible technique that could solve this would be the use of an anti-heparin agent. This could be applied to any contaminated sample to neutralize the effects of the heparin.

Hepzyme is a heparinase, which neutralizes therapeutic or contaminant heparin in clinical specimens (Forte *et al*., 2000).

Adding Hepzyme to a non heparin contaminated sample did not affect its clotting screen (PT and APTT) (Table 6.8).

Normals	PT(seconds)	APTT (seconds)
1	12.9	27.1
1+ Hepzyme	12.9	27.1
2	12.1	23.7
2 + Hepzyme	12.2	23.9
3	13.3	26.8
3+ Hepzyme	13.4	27.2
4	12.1	29.7
4+ Hepzyme	12.4	28.7
5	11.9	27.3
5+ Hepzyme	12.3	27.6
6	11.5	25.6
6+ Hepzyme	11.7	25.6
7	12.3	26.6
7+ Hepzyme	12.6	26.0
8	13.1	28.3
8+ Hepzyme	13.1	28.5
9	12.4	27.3
9+ Hepzyme	12.7	26.5
10	12.7	26.6
10+ Hepzyme	12.9	26.6

Table 6.8. Clotting Screens for 10 normal samples with and without Hepzyme. Abbreviations: prothrombin time (PT), Activated Partial Thromboplastin Time (APTT).

Hepzyme is not only effective with heparin but also with Low molecular weight heparins (LMWH) (Fragmin, Tinzaparin, Clexane) and Danaparoid Sodium (Table 6.9.).

Type of Heparin	Anti-Xa Level	PT(seconds)	APTT (seconds)
Normal PPP	0.00	12.9	36.0
PPP + Hepzyme	0.00	13.3	36.8
PPP +Heparin	1.02	14.4	201.9
PPP+Heparin + Hepzyme	0.00	13.1	33.8
PPP+Fragmin	1.00	17.0	171.2
PPP+Fragmin + Hepzyme	0.00	13.5	39.0
PPP+Tinzaparin	1.02	19.4	182.9
PPP+Tinzaparin + Hepzyme	0.00	13.5	36.2
PPP+Clexane	1.09	16.4	117.6
PPP+Clexane + Hepzyme	0.02	13.8	37.7
PPP+Danaparoid	1.07	15.7	119.1
PPP+Danaparoid + Hepzyme	0.00	13.6	38.4
PPP+Atrixa	>1.50	15.1	58.6
PPP+Atrixa + Hepzyme	0.12	14.1	47.2

Table 6.9. Effects of Heparin with or without Hepzyme on the PT and APTT. Abbreviations: platelet poor plasma (PPP), prothrombin time (PT), Activated Partial Thromboplastin Time (APTT).

The CAT is an assay that is very sensitive to heparin. It is able to detect minimal residues of heparin in a sample and can be used to monitor the use of any anticoagulant in an accurate manner (Gatt *et al*, 2008).

By using the CAT it was possible to visualize how Hepzyme could neutralize the effects of different types of heparins (Table 6.10.)

Sample Type	Lag time (min)	ETP (nmol)	Peak (nmol.min)	TT Peak (min)
Normal PPP	2.89	1466	104.51	8.24
PPP + Hepzyme	2.89	1335.5	90.08	8.07
PPP+Heparin	10.91	42.5	2.21	18.43
PPP+Heparin + Hepzyme	2.56	1411.5	103.96	7.40
PPP+Fragmin	7.23	141	6.34	15.08
PPP+Fragmin + Hepzyme	2.72	1354.5	97.46	7.90
PPP+Tinzaparin	8.24	60.5	2.63	19.43
PPP+Tinzaparin + Hepzyme	2.89	1393.5	101.54	7.74
PPP+Clexane	6.07	180.5	8.80	13.75
PPP+Clexane + Hepzyme	2.89	1299.5	93.42	8.24
PPP+Danaparoid	7.57	123.5	5.19	16.09
PPP+Danaparoid + Hepzyme	3.23	786	50.35	11.08
PPP+Atrixa	13.08	101	3.41	33.63
PPP+Atrixa + Hepzyme	2.89	1173	84.9	8.40

Table 6.10. Heparin with or without Hepzyme using the CAT. Abbreviations: platelet poor plasma (PPP), endogenous thrombin potential (ETP), time to peak (TT Peak)

To illustrate how Hepzyme neutralizes Heparin, the following graphs show the CAT assay run on samples containing different types of Heparin with and without the addition of Hepzyme. (Fig. 6.6., Fig. 6.7., Fig. 6.8., Fig. 6.9. and 6.10.).

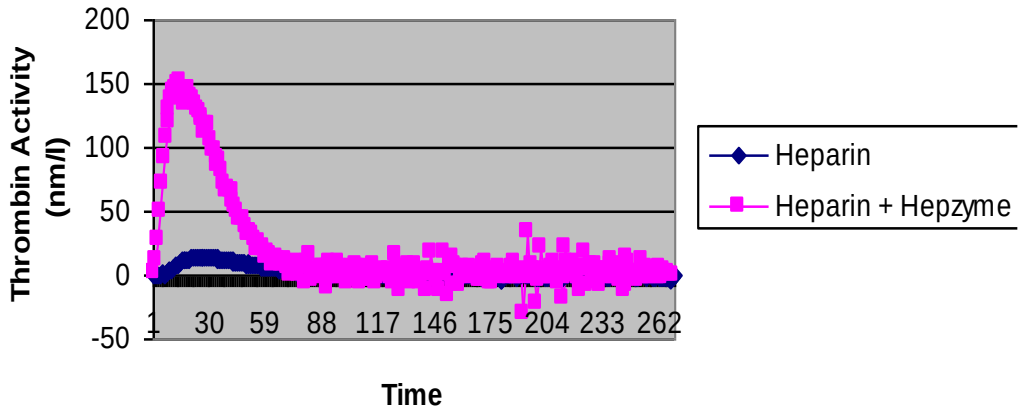

Figure 6.6. The effects of Heparin in the presence & absence of Hepzyme on the ETP.

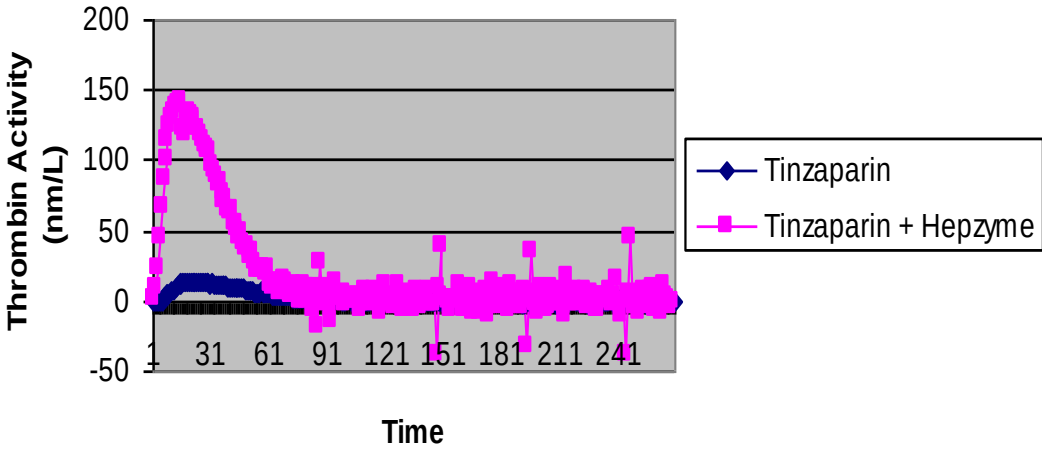

Figure 6.7. The effects of Tinzaparin in the presence & absence of Hepzyme on the ETP.

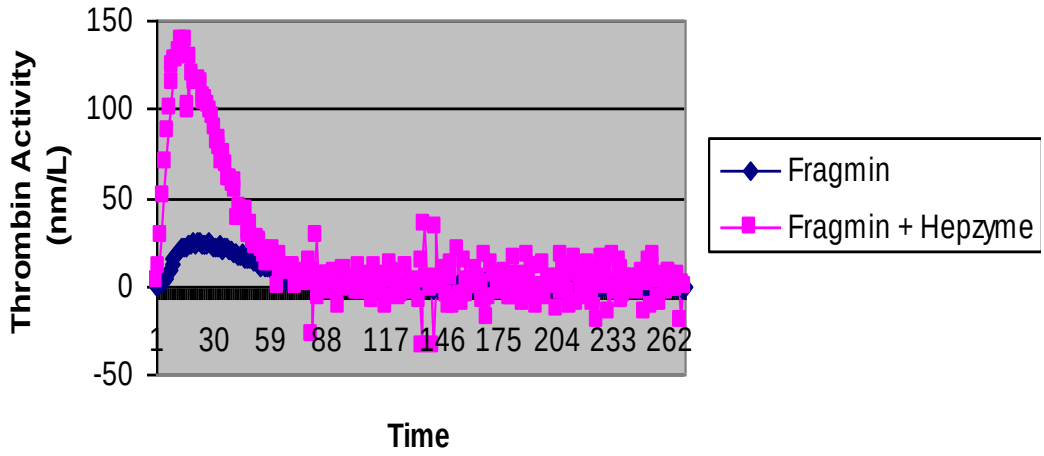

Figure 6.8. The effects of Fragmin in the presence & absence of Hepzyme on the ETP.

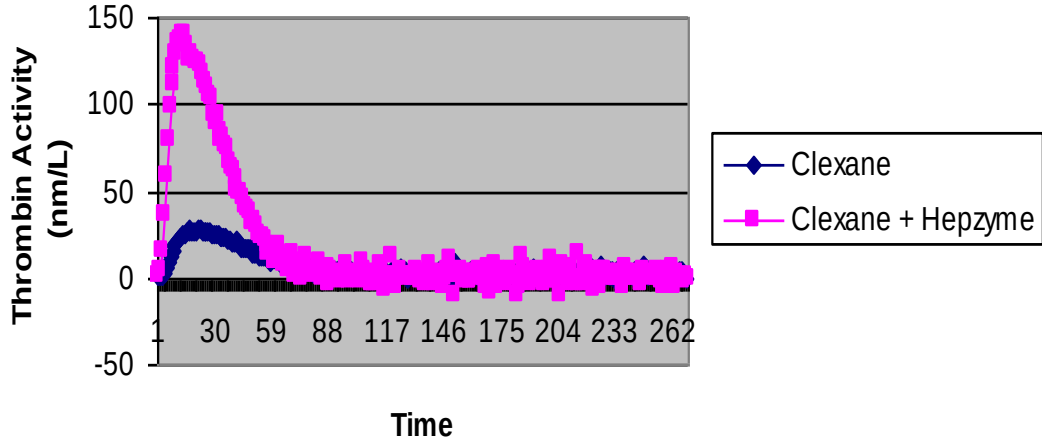

Figure 6.9. The effects of Clexane in the presence & absence of Hepzyme on the ETP.

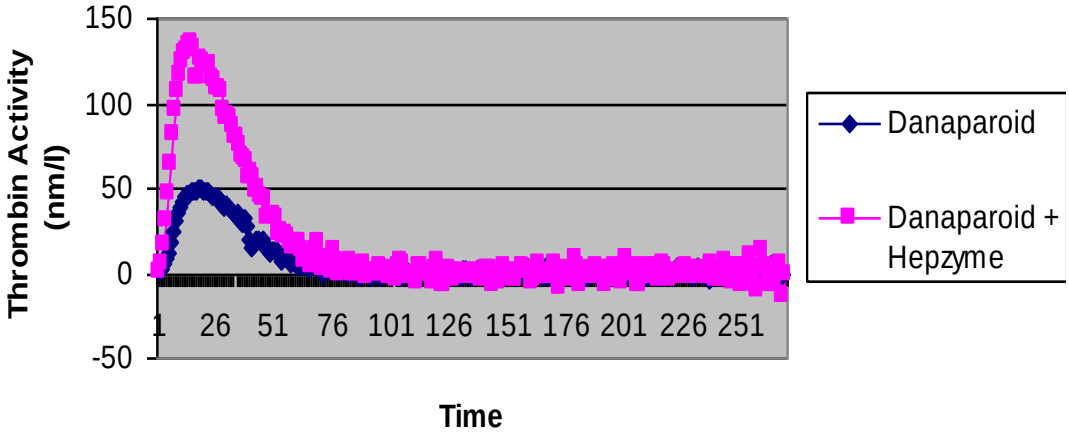

Figure 6.10. The effects of Danaparoid in the presence & absence of Hepzyme on the ETP.

The above experiments show that Hepzyme treatment could be used in the ICU patients for assessment of samples using CAT under hypothermia and acidosis and would enable meaningful results on a greater proportion of patients (only 10/17 (59%) can be used in the absence of heparin neutralisation).

7. INTRODUCTION

Massive bleeding in patients with major trauma is the most common cause of in-hospital mortality during the first 48 hours. Following admission the highest mortality occurs in patients with hypothermia, acidosis, and coagulopathy. (Patt *et al*, 1988). This combination is commonly referred to as the "lethal triad of trauma." Although the lethal triad has been recognized for a decade, the underlying mechanisms related to the development of coagulopathy are not fully understood.

Clotting Screens, such as the APTT, PT, and thrombin time, are prolonged when assays are performed at patient temperature in hypothermic patients or plasma cooled *in vitro*.

7.1 Activated partial thromboplastin time and prothrombin time

The PT and APTT assays are often used as screening tests and surrogate markers of coagulation factor levels in trauma settings. Testing the PT and APTT in this study under hypothermic and acidotic conditions showed and confirmed what has already been described by several groups around the world, i.e. that under hypothermic and /or acidotic conditions the PT and APTT experience a time prolongation (Breen *et al*., 1988; Reed, 1990; Gubler *et al*, 1994; Armand *et al*., 2003).

Hypothermia significantly prolonged the PT by more than 5 seconds ($p<0.01$) and the APTT for almost 2 seconds on PPP ($p<0.01$), (Table 4.1.). PRP APTT was also prolonged under hypothermic conditions for more than 8 seconds ($p<0.01$), (Table 4.2.). Acidosis prolonged the PT for almost 3 seconds and the APTT for more than 36 seconds on PPP (Table 4.1.). PRP APTT was also delayed by 29 seconds (Table 4.2.). These results showed that either hypothermia or acidosis have an effect on the coagulation process in vitro, by delaying PT and APTT clotting times.

Hypothermic and acidotic conditions alone significantly prolonged PT and APTT. When hypothermic and acidotic conditions were combined, the PT/APTT time delays were even longer, suggesting a possible cumulative effect of the two conditions over the coagulation process. PT was prolonged by more than 9 seconds ($p<0.01$) and the APTT by 58 seconds on PPP ($p<0.01$) under the combined conditions of acidosis and hypothermia (Table 3.1.). In addition, the PRP APTT showed a delay of 44 seconds ($p<0.01$), (Table 3.2.).

The progressively prolonged PT and APTT times *in vitro* under hypothermic and/or acidotic conditions suggest that unless temperature and/or pH are corrected a bleeding tendency will be observed *in vivo*.

7.2 Calibrated Automated Thrombogram

As the CAT assay is a fluorometric rather than a clot based technique, it is possible to continue with the measurement after the clot is formed. This is an important difference between the CAT and the standardised clotting screens such as PT and APTT. When clot formation occurs the clot tests stop measuring but for the CAT assay this is just the beginning of the assay which is called lag time. After the lag time the system will carry on with the measurement until the sample runs out of thrombin (Section 1.7.1.).

The use of two different types of plasma samples, namely PPP and PRP demonstrated that some parameters were consistent and behaved equally while others did not correlate nor follow any particular trend. The lag time and the ETP followed the same trend for both types of plasma samples. lag time on PPP, however was prolonged under hypothermic conditions by 0.46 minutes (The lag time at 31°C /pH 7.35 was 8.37 minutes (Table 4.3.)). ETP on PPP increased under hypothermic conditions from 1777nmol to 2638nmol of thrombin (Table 4.3.), whilst peak, time-to-peak and velocity did not show any correlation between PPP and PRP sample measurements.

With the CAT methodology there seems to be a disparity between some of the results obtained. In general, it seems that some parameters (lag time and ETP) are affected by both conditions on its own but when both are combined (hypothermia + acidosis) there is a cumulative effect which exaggerates the changes. Lag time experienced a 2.55 minutes delay on PPP (Table 4.3.) and 2.3 minutes delay on PRP (Table 3.5). ETP increased by 1075nmol of thrombin (Table 4.3.) and 845.2 nmol of thrombin (Table 3.5) on PPP and PRP respectively.

The observed delays in lag time, which correlated with worsening conditions, supports the findings of the standard laboratory assays and previous literature (Tables 4.3 & 4.5. for CAT lag times and tables 4.1 & 4.2 for PT/APTT). The data started to diverge from the published perception of hypocoagulability based upon laboratory data when the ETP was examined.

The ETP began to increase when temperature was reduced (861nmol on PPP and 435.55nmol on PRP) (Tables 4.3. and 4.5.)) or pH decreased (208nmol on PPP and 276.2nmol on PRP) (Tables 4.3. and 4.5.)). The cumulative effect of acidotic and hypothermic conditions was also observed in the overall ETP (1075nmol on PPP and 845.2nmol on PRP) (Tables 4.3. and 4.5.)). These particular observations do not fit with any of the previously published literature, where a hypocoagulable state is always seen under abnormal conditions, such as hypothermia and acidosis.

These CAT results under abnormal conditions suggest that in spite of having a delayed lag time, which will indeed present as a bleeding risk to the patient, once the thrombin burst occurs elevated levels of thrombin will be generated. This could clinically manifest as a thrombotic rather than haemorrhagic risk.

7.3 ROTEM

Generally speaking, the results obtained from the ROTEM® methodology indicate a clear tendency for a coagulopathy in critical conditions, namely acidosis and hypothermia. The times to clot were very prolonged, the clot formation rates were decreased and clot stability worsened.

There was a close similarity between the results from the whole blood NATEM® Assay and the ones performed on the PPP with the EXTEM® assay was good (i.e. increments on CFT were observed with both assays when hypothermic and acidotic conditions were used). CFT=245% increase on EXTEM® (Table 4.7.) and CFT=207% increase on NATEM® (Table 4.8.).

All of the measured parameters confirmed that when acidotic and hypothermic conditions were combined that haemostasis was affected and a hypocoagulable picture was observed *in vitro*, with a delay in clot formation and a weaker clot formed (i.e. MCF decreased to 43.6 mm from 54.8mm under physiological conditions on the NATEM® assay (Table 4.8)).

AUC values, which were decreased at critical conditions (i.e. The 37/7.35 AUC was 5494.2nmol of thrombin compared to the 31/6.9 AUC, which was 4460.4nmol of thrombin on the NATEM® assay (Table 4.8)) were also consistent with this conclusion.

These findings are in agreement with previously published data on acidosis (Engström *et al*. 2006) and hypothermia effects on coagulation using Thromboelastometry (Kheirabadi *et al*., 2007; Dirkmann D *et al*., 2008; Rohrer *et al*.,1992).

It is interesting to note that the hypercoagulable findings of the CAT assay are not reflected in this “global test” of haemostasis. This is presumably due the nature of the end point detection. When fibrin polymerisation is used as the endpoint as in the ROTEM the effect of prolonged thrombin generation does not influence the assay. A similar finding to that experienced in the PT and

APTT. It is only when assess thrombin as the end-point that the anomaly becomes evident.

7.4. REFERENCE RANGES

By studying how samples obtained from healthy volunteers would perform under different conditions of temperature and/or pH it is possible to establish their CAT assay ranges. These ranges could be used as standards for future comparisons with any given sample at any specific condition.

The CAT assay demonstrated that hypothermia and acidosis alone induced a hypocoagulable pattern initially, until the thrombin burst occurs. Then the thrombin generated, as defined by the ETP, becomes higher as the conditions worsen. *In vitro* analysis using healthy volunteers demonstrated that hypothermic and acidotic conditions produced a cumulative effect when used in combination.

Higher body temperatures were also tested *in vitro* with the CAT assay. This showed the opposite effect of hypothermia. The lag times shortened as the temperature was increased (i.e. Lag time at hyperthermia shortened by more than a minute at 41°C; LAG = 5.03 min at 41°C, ($p<0.01$) compared to 37°C (Table 5.4.)) and the ETP became lower as the assay temperature rose.

The third parameter used to establish the reference ranges was the peak height of the CAT curve. The peak showed some correlation with the ETP but the changes produced by the different temperatures and pHs were not significant. The only significant peak values observed were at 31°C ($p<0.05$) and at 41°C ($p<0.01$).

7.5. ICU SAMPLES

Acidosis, hypothermia, and coagulopathy have been identified as the lethal triad for patients admitted to ICU. Recent studies report most severely injured patients to be coagulopathic on admission and that traditional massive transfusion practice grossly underestimates needs (Gonzalez *et al*., 2007). Management of coagulopathy is based on blood component replacement therapy. However, there is a limit to the level of haemostasis that can be restored and that can be predictive of mortality (Spahn *et al*, 2005).

The present study showed that standard investigations in a haemostasis laboratory will not be suitable for any patient who has body hypothermia or acidosis. Laboratory tests will underestimate the patient's real condition due to default temperature and pH correction when analyzed.

It is common practice that coagulation analyzers are set to a standard temperature of 37°C, and this will consequently not take into account the original body temperature of the patient. Sample buffering will also result in tests being analyzed at a physiological body pH without allowance for the patient's original blood pH.

By comparing the CAT results obtained from the ICU patients in their original hypothermic and acidotic state, with the CAT results from these patients with corrections applied for temperature and pH, it was possible to see how these corrections affected the results.

The results changed dramatically with the temperature and pH corrections. The lag times shortened by more than a half, the ETP decreased by around 300nmol.min of thrombin, the peak was slightly lower and the time-to-peak was also reduced in magnitude (Table 6.2.). Temperature and pH corrections resulted in results that significantly different. At this point, interpretation of these results is critical for the diagnosis but by applying temperature and pH corrections the results were changed to almost reflect normality.

7.5.1. ICU CAT results compared to CAT Reference ranges

To compare a CAT result from any apparently healthy person with that of an ICU patient two possible approaches were chosen. First, they could be compared at standardised conditions or second, they could be compared at the original ICU patient temperature and pH.

Measuring the CAT profile at a temperature of 35°C and a pH of 7.1 (35°C /pH 7.1) indicated that ICU samples demonstrated hypo-coagulable profile, but when conditions were standardised, the pattern seems to show an opposite situation.

Lag times obtained from ICU patients at 37°C /pH 7.35 suggested a hyper-coagulable state. ETP seems to be closer to the reference range at 37°C /pH 7.35 than at 35°C /pH 7.1. In both cases, ICU patients tended to have a lower ETP than healthy volunteers. The peak height of the ICU patients at 35°C /pH 7.1 was within the reference range observed under the same conditions. When tested at 37°C /pH 7.35 ICU peak values were below the lower limit of the reference range. This indicates that ICU samples have a hypo-coagulable tendency when measured at corrected pH and temperature but seem to be “normal” when tested at their original conditions.

The role of hypothermia and acidosis during sample analysis, would indicate that establishing reference ranges for any given temperature or pH could be beneficial for the diagnosis and management of ICU patients.

7.6. Problem solving

7.6.1. pH

After performing hundreds of measurements during this project on pH modified samples, it was possible to conclude that pH manipulation, if not done carefully, could completely alter the results. The main problem encountered when modifying the pH was coagulation activation. To avoid

sample coagulation cascade activation, a duplicate sample had always to be used for pH manipulation to allow the amount of base or acid added to be calculated. The same volume could then be added to another sample for experimental purposes. By doing this, experimental samples were never exposed to the pH meter glass probe, which was found to be a possible cause of activation of the plasma (Table 3.1.).

7.6.2. Cold activation

To avoid the freezing and thawing effects (Norda *et al*., 2008; Palmer *et al*., 1982), this study was performed on fresh samples. The only way to achieve this was by bleeding the healthy volunteers each time before analysis was performed and analysing the samples from ICU at the time of collection.

7.6.3. HEPARIN

Due to problems with heparin contamination, some of the samples received from the ICU could not be measured on the CAT analyser and therefore, the data was lost and more importantly valuable samples were wasted.

Heparin is an anticoagulant with several effects on the coagulation cascade. The most important one is the increase in the thrombin inhibitory potency of ATIII by 1000 fold (Blauhut *et al*., 1980). ATIII inhibits thrombin + FIXa, FXIa & FXIIa. Heparin contamination prolongs the APTT, and to a lesser extent the PT and also affects thrombin generation as measured by the CAT assay, which is much more sensitive than traditional coagulation tests to heparin within the sample (Gatt *et al*., 2008).

A possible technique adaptation that could remove this problem would be the use of an anti-heparin agent. This could be applied to any contaminated sample to neutralize the effects of the heparin. Hepzyme is a heparinase, which neutralizes therapeutic or contaminant heparin in clinical specimens (Forte *et al*., 2000). Adding Hepzyme to a non heparin contaminated sample however did not affect its clotting screen (PT or APTT), (Table 6.8).

The CAT procedure is an assay that is very sensitive to Heparin. It is able to detect minimal residues of heparin in a sample and can be used to monitor the use of any anticoagulant in an accurate manner (Gatt *et al*, 2008).

By using the CAT procedure it is possible to visualize how Hepzyme can neutralize the effects of different types of heparins (Table 6.10.). Data from this study showed that Hepzyme can be effective against several types of heparinoids, such as heparin, Fragmin, Tinzaparin, Clexane, Danaporoid and Atrixa (Table 6.10.).

7.7. CONCLUSIONS

Both hypothermic and acidotic conditions contribute to coagulopathy, and that their combination has additive effects.

CAT results produced under hypothermic and/or acidotic conditions suggest that in spite of having initially a delayed lag time, once the thrombin burst occurs it will generate more thrombin which will increase the overall ETP. Therefore once the initial lag time has passed there is a potential hypercoagulable situation.

Traditional clotting screens are not the most suitable assays to be performed for the study of coagulation in critical care patients, because they only measure the initiation of the coagulation process.

Global assays, such as thromboelastometry and CAT are better suited for the clinical investigation in the ICU but an understanding of the nature of the end-point of the tests is essential when interpreting the results. After clot formation the measurement continues with global assays and valuable information is obtained, such as clot firmness and stability or how much thrombin is produced and for how long.

Although these standard clotting tests are performed at 37°C and physiological pH, *in vitro* testing of any given sample should ideally be performed at the patient's own body temperature and pH as well as under the corrected conditions. This will give a picture of what is happening now compared to what will happen when the hypothermia/acidosis is corrected.

7.8. Future work

This study has shown the effects of hypothermia and acidosis on the haemostatic process and how different assays can give results that not always follow the same pattern.

To achieve a better understanding of how these abnormal conditions affect coagulation, it would be interesting to look at other coagulation factor levels during hypothermia and with acidosis. Coagulation factors such as factors IX, X and XI; Prothrombin levels, Protein C activation pathway and Antithrombin levels and functionality.

During this study it has been very difficult to get hold of samples from ICU on a regular basis. Looking into how to improve the supply of samples from ICU would be very beneficial for this investigation.

Further studies on the effects of extensive use of Hepzyme on any heparin contaminated sample would be recommended.

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- www.stago-us.com

- www.thrombinoscope.nl

dmu.ac.uk
DE MONTFORT UNIVERSITY
LEICESTER

8th January 2007

Mr Luis Figueiredo De Sousa
Trainee Clinical Scientist
Haematology Department Box 234,
Level 3,
Addenbrookes Hospital,
Hills Road,
Cambridge.
CB2 2QQ

Dear Mr Figueiredo De Sousa,

Re: Ethical approval application for 'Thrombin Generation in Critically Ill Patients'

I am writing regarding your application for ethical approval for a research project titled 'Thrombin Generation in Critically Ill Patients'. This project has been reviewed in accordance with the Operational Procedures for De Montfort University Faculty of Health and Life Sciences Research Ethics Committee. These procedures are available from the Faculty Research and Commercial Office upon your request.

I am pleased to inform you that ethical approval has been granted by Chair's Action for your application . This will be reported at the next Faculty Research Ethics Committee, which is being held on 7th February 2007.

Should there be any amendments to the research methods or persons involved with this project you must notify the Chair of the Faculty Research Ethics Committee immediately in writing. Serious or adverse events related to the conduct of the study need to be reported immediately to your Supervisor and the Chair of this Committee. Also, The Faculty Research Ethics Committee should be notified by e-mail to HLSFRO@dmu.ac.uk when your research project has been completed.

Yours sincerely,

Professor Paul Whiting
Chair
Faculty of Health and Life Sciences
Research Ethics Committee

Professor G Grant
Dean

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Peterborough & Fenland Local Research Ethics Committee
Victoria House
Capital Park
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Cambridge
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Telephone: 01223 597653
Facsimile: 01223 597645

31 August 2005

Dr Vilas U Navapurkar
Consultant in Intensive Care Medicine and Anaesthesia
JVF ICU, Box 17
Addenbrookes Hospital
Hills Road
Cambridge
CB2 2QQ

Dear Dr Navapurkar

Full title of study: **Thrombin Generation in Critically Ill Patients**
REC reference number: **05/Q0106/63**

Thank you for your email of 26 August 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair and the Administrator, acting under delegated authority.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

The favourable opinion applies to the research site listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application (Lock code AB/48979/1) - signed 03/08/2005		07 June 2005
Investigator CV		(None Specified)
Protocol	1	07 June 2005
Covering Letter		(None Specified)
Summary/Synopsis	1	07 June 2005
Compensation Arrangements: Email from R& D, Addenbrooke's Trust re indemnity		27 July 2005